# NON-KERATINS OF HUMAN HEAD HAIR

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The natural state of human head hair is constantly interfered with by cutting, washing and grooming, and it is therefore possible that some or most of the non-keratins (water-soluble organic compounds in keratinous structures) are leached out or destroyed in these grooming processes and that, consequently, analytical results could be doubtful or valueless (Bolliger and Gross, 1952). In spite of this possible source of error, however, about 40 different specimens of head hair obtained from normal subjects, hospital patients and cadavers were examined for at least two, but frequently for more, non-keratinous constituents such as total nitrogen, urea, ammonia, uric acid, amino acids, pentose, and total reducing substances before and after hydrolysis. These results are presented herewith and discussed.

## METHODS.

Human head hair was obtained from normal adults, from hospital patients, many of whom were undergoing head operations, from cadavers, and from normal and hospitalized children. These specimens were boiled out with water, as described previously, and the aqueous extract was analyzed using methods employed in previous investigations (Bolliger and Gross, 1952, 1952a, 1953). After the removal of ammonia, amino acids present in the aqueous extract were determined by Folin's method (1922) before and after hydrolysis. Hydrolysis was executed by refluxing the aqueous extract for 24 hours with 8 volumes of 8N sulphuric acid, followed by neutralizing with barium carbonate. The precipitated barium sulphate was removed by centrifugation and was washed with water. The supernatants were concentrated to the original volume. In some instances half of the specimen of cut hair was washed with soap and water at 35–40° C., imitating the usual technique of hair-washing, and compared with the unwashed half.

The results in all cases are expressed in milligrams of the non-keratin concerned per 100 gm. dry weight of hair.

## RESULTS.

*Total nitrogen.* The total nitrogen content of the aqueous extract of 24 specimens of head hair from adult normals and from adult patients without any known renal involvement ranged from 72 to 245 mg. p.c. (Table 1). In 15 of

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TABLE 1.

*Findings on hair of adults.*

| | | Age | Sex | Total N mg. p.c. | Urea N mg. p.c. | $NH_3$ N mg. p.c. | Uric acid N mg. p.c. | Amino acid N mg. p.c. | Amino acid N H* mg. p.c. | Reducing substances** mg. p.c. | Reducing substances** H* mg. p.c. | Pentose mg. p.c. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | A.B. | 52 | M | 93 | 8 | 18 | 8 | | | | | | Normal |
| 2. | A.B. | 53 | M | 79 | 0 | 14 | | | | | | | Normal |
| 3. | A.B. | 54 | M | 90 | 0 | 20 | 9(6) | | | 63 | 67 | 30 | Normal |
| 4. | A.B. | 55 | M | 84 | | | 10(6) | 9 | 12 | 47 | 47 | | Normal |
| 5. | A.B. | 56 | M | 78 | 6 | 23 | 8 | 12 | 14 | 121 | 131 | 34 | Normal |
| 6. | T.P. | 31 | F | 72 | 0 | 1 | | | | 294 | 334 | | Normal |
| 7. | H.D. | 32 | F | 78 | | | | | | 61 | 65 | 23 | Normal |
| 8. | D.B. | 47 | F | 83 | 1 | 22 | 4 | 5 | 11 | 82 | 84 | 30 | Normal |
| 9. | E.L. | 34 | F | 83 | | | 12(6) | | | | | | Astrocytoma |
| 10. | R.H. | 27 | F | 84 | | | | | | 82 | 82 | | Normal |
| 11. | B.B. | 26 | F | 91 | 0 | 24 | 16 | 9 | 13 | 48 | 53 | 20 | Normal |
| 12. | R.D. | 31 | M | 95 | | | 6 | | | 42 | 57 | 41 | Normal |
| 13. | Y.L. | 41 | F | 97 | | | 8(6) | | | | | | Normal |
| 14. | J.L. | 47 | F | 98 | 5 | 18 | 9 | | | 44 | 70 | 17 | Fits |
| 15. | L.I. | 62 | F | 100 | 7 | 4 | 10 | | | 535 | 595 | 34 | Neuralgia |
| 16. | M.A. | 32 | M | 107 | | | | | | 38 | 38 | 13 | Diabetes |
| 17. | T.C. | 27 | F | 122 | 8 | 13 | 10(5) | 10 | | 235 | 235 | 18 | Aneurism |
| 18. | B.B. | 32 | F | 125 | 9 | 30 | 11 | | | 74 | 121 | | Glioma |
| 19. | A.S. | 25 | M | 141 | 11 | 45 | 14 | 11 | | 34 | 55 | 29 | Brain abscess |
| 20. | A.L. | 61 | M | 168 | 0 | 33 | 14(11) | | | 44 | 47 | 17 | Gout |
| 21. | J.T. | 60 | M | 169 | 10 | 33 | 12 | 17 | 35 | 60 | 63 | 17 | Cystic brain tumour |
| 22. | G.T. | 31 | M | 184 | 16 | 52 | 13 | 23 | | 61 | 67 | 33 | Astrocytoma |
| 23. | L.T. | 34 | F | 186 | 0 | 59 | | | | 54 | 63 | 31 | Glioblastoma |
| 24. | J.G. | 28 | F | 197 | 30 | 22 | 7 | 18 | | | | | Moderate Nephritis |
| 25. | A.M. | 27 | M | 245 | 48 | 84 | 10 | | | 50 | 59 | 30 | Subdural haemorrhage |
| 26. | A.B. | 41 | M | 305 | | | 19(14) | | | | | | Gout and moderate nephritis |
| 27. | G.B. | 50 | M | 357 | 40 | 133 | 15 | 74 | 74 | 117 | 103 | 32 | Subdural haemorrhage and nephritis |
| 28. | A.G. | 35 | M | 604 | 202 | 116 | 34(28) | | | 86 | 86 | 46 | Gout and nephritis‡ |
| 29. | W.T. | 84 | M | 861 | 188 | 196 | 17(14) | | | | | | Chronic nephritis‡ |

H*=after hydrolysis.
**=determined as glucose.
( )=Bracketed figures in "Uric acid" column represent values obtained by uricase method.
‡=deceased.

these specimens, of which 12 were obtained from normals, the total nitrogen content did not exceed 100 mg. p.c. Five of these specimens came from a healthy middle-aged male (A.B.) whose hair was examined over a period of five years for a number of non-keratin constituents. In this instance the hair was combed and washed superficially each day and washed more intensively under the shower about two to three times a week.

The specimens of head hair containing from 122 to 245 mg. p.c. of total nitrogen in the aqueous extract were all from hospital patients. The head hair of two deceased patients suffering from chronic severe renal insufficiency (Cases 28, 29, the first case complicated by gout) yielded total nitrogen values of 604 and 861 mg. p.c. respectively. A comatose patient (27) with a subdural haemorrhage and a history of severe alcoholism and moderate renal insufficiency yielded 357 mg. p.c. of total non-keratin nitrogen.

In 15 specimens from children between 1 and 12 years of age the total non-keratin nitrogen content varied from 122 to 286 mg. p.c. (Table 2). In a child of about 8 months of age it reached a value of over 400 mg. p.c.

TABLE 2.

*Findings on hair of children.*

| | Age Years | Sex | Total N mg. p.c. | Urea N mg. p.c. | $NH_3$ N mg. p.c. | Reducing substances mg. p.c. | H* mg. p.c. | Pentose mg. p.c. | |
|---|---|---|---|---|---|---|---|---|---|
| Y.C. | 0·7 | F | 436 | 58 | 60 | | | 85 | Normal |
| M.L. | 1·1 | F | 307 | | | | | 117 | Normal |
| T.B. | 6 | M | 208 | 35 | 53 | 42 | 42 | 22 | Concussion |
| J.B. | 10 | F | 160 | | | 43 | 44 | 20 | Cranio-pharingeoma |
| 14 children | 2–12 | | 122–286 | | | | | 19–73 | Normal |

H*=reducing substances after hydrolysis.

*Ammonia and urea.* Ammonia was always present in the aqueous extract of hair. With total nitrogen values up to 125 mg. p.c., the ammonia nitrogen content fluctuated between 1 and 22 mg. p.c. It increased with increasing total nitrogen content, reaching 196 mg. p.c., in a severe nephritic of 84 years of age. The urea content fluctuated between 0 and 16 mg. p.c., and in five adults was found to be nil. Urea values over 16 mg. p.c. were only found accompanying a total nitrogen value of over 200 mg. p.c. With only two exceptions (Cases 15 and 25) the ammonia nitrogen content was higher than the urea nitrogen content.

*Amino acid nitrogen.* About 10 to 20 p.c. of the total nitrogen consisted of amino acid nitrogen which rose markedly when the total nitrogen increased over 200 mg. p.c. (Case 27). After hydrolysis of the aqueous extract, the amino acid nitrogen content frequently increased.

*Uric acid.* With the exception of three nephritic patients (Cases 27, 28, 29) and three with gout (Cases 20, 26, 28), uric acid as determined by the "direct" method of Folin varied from 4 to 12 mg. p.c. With the uricase method, which was only used on five specimens from non-nephritics, the uric acid range was 4 to 6 mg. p.c. Nephritic and gout patients yielded higher uric acid values with both the "direct" and the uricase method.

*Reducing substances.* In 19 of 24 determinations (Tables 1 and 2) the reducing substances of the aqueous hair extract ranged from 34 to 86 mg. p.c., one of the lowest values being obtained from the hair of a severe diabetic who had been treated with insulin for over 20 years (Case 16). The five values exceeding this range were found amongst normal subjects as well as patients. The highest (540 mg. p.c.) was obtained from the white hair of a woman, 62 years of age, suffering from neuralgia (Case 15).

On hydrolysis of the aqueous hair extracts the reducing substances increased materially (i.e., more than 10 mg. p.c.) in only six of 26 specimens examined. This indicates that polysaccharides are frequently absent, or present only in comparatively small amounts in human hair. The iodine test for glycogen became faintly positive in those specimens where the reducing substances increased materially after hydrolysis.

Pentose ranged from 13 to 46 mg. p.c., the lowest pentose content being obtained from the same diabetic who had one of the lowest amounts of reducing substances before and after hydrolysis. The pentose did not increase when exceptionally high amounts of reducing substances (Cases 6, 15, 17) were encountered. Usually pentose represented about half of the reducing substances.

*Washing experiments.* Washing cut hair, which appeared to have had the usual care before cutting, with soap and water at 35–40° C. previous to extraction of non-keratins did not materially diminish the yield of non-keratins as shown in Table 3. The only exception was a patient aged 62 years (Case 15) where the

TABLE 3.

*The influence of hair-washing (water 35–40° C. and soap) on non-keratins of groomed female human hair.*

| Age | Total N mg. p.c. | Urea N mg. p.c. | $NH_3N$ mg. p.c. | Uric acid mg. p.c. | Reducing substances mg. p.c. | H* mg. p.c. | Pentose mg. p.c. | |
|---|---|---|---|---|---|---|---|---|
| J.C. 47 | 98 | 5 | 18 | 9 | 44 | 70 | 17 | Before |
| | 82 | 4 | 16 | 9 | 38 | 57 | 14 | After |
| L.I. 62 | 100 | 7 | 4 | 10 | 540 | 605 | 34 | Before |
| | 62 | 0 | 1 | 10 | 544 | 627 | 18 | After |
| J.B. 10 | 156 | | | | 43 | 43 | 19 | Before |
| | 164 | | | | 42 | 46 | 21 | After |
| B.B. 26 | 91 | 0 | 24 | 8 | 40 | 40 | 20 | Before |
| | 90 | | | 8 | 38 | 41 | 19 | After |

H*=reducing substances after hydrolysis.
Before=before washing. After=after washing.

total nitrogen content fell after washing from 100 to 62 mg. p.c. although the high content of reducing substances was not diminished. Neither the total nitrogen content (160 mg. p.c.) nor the reducing substances in the cut hair of a girl aged 10 years were diminished by washing.

## DISCUSSION.

The processes concerned in the usual washing of head hair and those which were employed in these investigations for the complete dissolving out of non-keratins from hair have some similarity, although in the analytical procedures the non-keratins were boiled out of the hair during an hour or more whilst toilet grooming uses temperatures up to about 40° C. at most and usually much less vigorous procedures. Certainly a fraction of the non-keratins can be expected to be leached out by toilet washing but, in spite of the great variety of grooming methods resorted to by different individuals, the analytical results yielded by the aqueous extract of human hair are by no means valueless.

It appears that the head hair of normal adults, male or female, exposed to the usual amount of grooming contains about 100 mg. p.c. or less of total non-keratin nitrogen. The fluctuations over a period of five years in at least one individual were apparently insignificant.

The majority of the hospital patients examined had a total non-keratin nitrogen content of 122 to 186 mg. p.c. It is not yet possible to state definitely if these higher values are due mainly to disease or to diminished care and washing of the head hair. Such higher values would be quite understandable in sick people who frequently also suffer from copious perspiration. In severe chronic nephritis (Cases 28 and 29) the very high total nitrogen values (600 to 800 mg. p.c.) are undoubtedly connected with the clinical condition.

A young non-nephritic patient (Case 25) suffering from subdural haematoma, accompanied by extreme restlessness, yielded the high total nitrogen content of 245 mg. p.c. At present no attempt can be made to explain this high value but, as suggested by his history, this patient may not have washed his hair for some years.

It is, of course, possible that the high nitrogen values as found in children compared with adults are due to less vigorous hair grooming; however, in an actual washing experiment on the cut hair of a girl aged 10 the total nitrogen did not diminish (Table 3). It seems that even with the wide variety of methods of hair grooming in common use a certain varying quantity of non-keratin nitrogen is maintained on the fibre and further marked diminutions do not usually take place when the cut hair is washed experimentally.

The total nitrogen content in the present series of hair specimens, excluding those from nephritic patients, is only about one-third of that found in toe-nails from "normals" (Bolliger and Gross, 1953). The more intensive grooming of

the head hair and its much greater surface leading to a much greater exposure to leaching out may explain the large difference.

However, there is a fundamental difference between the non-keratin ammonia nitrogen content of toe-nails and head hair which cannot be thus explained. In human toe-nails ammonia values seem to remain within a fixed range even in conditions of severe urea retention, and no value over 40 mg. p.c. was encountered (Bolliger and Gross, 1953a). In marked contrast to this the ammonia content of the head hair of nephritics exceeded 100 mg. p.c. and was also found to be above toe-nail level in five non-nephritic patients. Furthermore, it appears that grooming removes urea more readily than ammonia from hair, and in a number of instances no urea was found to be present while considerable amounts of ammonia could still be demonstrated. It is believed that the total absence of urea is an artefact brought about by intensive hair washing and that, primarily, urea is always present in the ungroomed hair of human subjects, just as it is always found in their toe-nails and in the hair fibres of other mammals.

On account of its simplicity, Folin's "direct" uric acid method was employed in the majority of determinations in preference to the more specific but more cumbersome uricase method (Bolliger and Gross, 1952b). Thus "normal" values for the "direct" method were established. At present it may be stated that in a quick survey the rapid "direct" method of Folin gives an indication of increased uric acid values, but the problem will be discussed more fully in a separate paper on non-keratin uric acid in gout.

It may be pointed out, however, that in spite of the low uricolytic index in man, the urate content of human hair and other keratinous appendages paradoxically is lower than that found in any other mammal examined (Bolliger and Gross, 1952, 1952a, 1953). Grooming is not responsible because urates are sparingly soluble and were not diminished by experimental washing.

The sum of those nitrogenous constituents which were quantitatively determined together with the considerable amino acid nitrogen fraction and an allowance for total creatinine and allantoin nitrogen still leaves about half of the non-keratin nitrogen unidentified. This larger fraction of undetermined nitrogen has been encountered in all the investigations on non-keratins and awaits further elucidation.

In the great majority of specimens the reducing substances showed but little variations either in normal subjects or in hospital patients. The values were usually well below those of the normal blood sugar level. No explanation can be given for the exceptionally high content of reducing substances in one individual, viz., 540 mg. p.c. (Case 15). It is of interest, however, that the reducing substances, even when present in large amounts, cling to the hair fibre quite tenaciously and are not leached out by washing experiments in the laboratory.

Of all the mammalian hair fibres examined human head hair yields the smallest percentage of non-keratins. This is partly due to the grooming habits of modern man, but, in spite of these, upper normal values can be perceived which apply to most individuals, viz., total nitrogen 150 mg. p.c., ammonia nitrogen 50 mg. p.c., uric acid 6 mg. p.c., by the uricase method and 12 mg. p.c. by the "direct" method. The water-soluble total nitrogen, urea, ammonia and uric acid of head hair are elevated in severe chronic renal insufficiency and uric acid is elevated in gout.

## SUMMARY.

Total nitrogen, ammonia, urea, uric acid, reducing substances before and after hydrolysis and pentose were determined on the aqueous extract of human head hair of normal children and adults, hospital patients and cadavers.

Notwithstanding the wide variety of pathological conditions and the varieties of hair grooming habits, pentose and total reducing substances were present within a narrow range in most specimens. Occasional very large amounts of reducing substances cannot be explained.

Total nitrogen in the aqueous extracts of hair of normals and of patients with customary grooming habits was far below general mammalian levels. In a number of patients and of children the total nitrogen values were higher than in normal adults. Outstandingly high total nitrogen content was encountered in lethal chronic renal insufficiency.

The ammonia nitrogen content rose in proportion to the increasing total nitrogen and was usually higher than that of urea nitrogen.

As with toe-nails, the uric acid content of hair was elevated in moribund patients with severe chronic renal insufficiency and in gout.

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# NON-KERATINS OF AVIAN AND MAMMALIAN SKIN FLAKES

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The human scalp produces horny flakes or scales which, when occurring in readily visible quantity, are referred to as scurf or dandruff (Pityriasis capitis). These scales are cast off from the cranial skin, which, like skin in general, constantly renews its surface. Where there is a dense growth of hair, the skin flakes formed on the scalp are retained, but on the hairless skin they are usually detached and quickly disappear. Similar scales are produced and retained to a large extent by fur-covered mammalian skin in general, and by the feather-covered skin of birds. All skin scales are keratinous in nature and contain, in addition to the insoluble keratin, considerable amounts of water-soluble organic substances, the so-called non-keratins. These skin-flake non-keratins were studied on a number of birds and mammals, excluding man, and were compared with the respective non-keratins of furs and feathers.

## METHODS.

The skin flakes were obtained by immersing and agitating clipped mammalian fur or plucked birds' feathers in ethyl or petroleum ether and filtering the resulting suspension. This washing process releases the fine flakes from the greasy fur or feathers to which they are attached. The skin flakes are collected on filter paper, washed with ether and dried. Frequently they are characterized by a grey silvery sheen.

Skin flakes were also obtained by immersing a sufficiently clean animal wholly or partly in ether. Prior to immersion, the surface of the animal may be cleaned by dry brushing and sponging with ether. Agitating the immersed animal, combing the fur and renewing the ether once or twice increased the yield. This method, which also removes the skin flakes still loosely attached to the skin, appears to be very satisfactory, particularly for small or short furred animals. By evaporating the ether in which the animal has been immersed, the dissolved sebum can be isolated.

Ethyl ether is usually preferable to petrol ether inasmuch as it releases the skin flakes more readily and the ensuing suspension filters more rapidly. In the rabbit only ethyl ether is satisfactory, petrol ether releases no skin flakes whatsoever. On the other hand, ethyl ether carries the danger of anaesthetic death for the experimental animal. Skin flakes may be purified by resuspending in ether and filtering. Solid impurities are usually heavier than the flakes and sink to the bottom of the vessel. Hairs still present in the skin flakes may be removed by sieving.

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The dried scales thus obtained were next extracted with boiling water as described previously in the case of furs, feathers, nails, etc. The extracts were then "deproteinized" with tungstic acid, and analysed for total nitrogen, ammonia, urea, uric acid, amino acids and reducing substances before and after hydrolysis. Desoxyribose and pentose content were ascertained on "non-deproteinized" extracts. These determinations were made by methods used previously (Bolliger and Gross, 1952, 1952a, 1952b and 1954).

Paper chromatography for amino acids was carried out on Whatman No. 1 filter paper, using the ascending technique. The solvent was water-saturated phenol containing a small quantity of 8-hydroxyquinoline. For two dimensional runs collidine saturated with water was used after developing with phenol. For the chromatographic detection of sugars the top layer of an ethyl-acetate-pyridine-water mixture 2:1:2 by volume was used. After drying, the paper was sprayed with 0·1 M aniline hydrogen phthalate dissolved in moist n-butanol and heated for 5 minutes at 105° C.

## RESULTS.

With the method of isolation described, skin flakes could be obtained from all the birds and mammals examined, but the amount varied greatly in different species.

Plucked feathers of young butcher birds (*Cracticus torquatus*) contained approximately 3 p.c. of skin flakes. The feathers of other birds contained smaller amounts and the plumage of the dollar bird (*Eurystomus orientalis*) yielded less than 0·1 p.c. of skin flakes. The skin flake content of cut mammalian fur was of a similar order and varied similarly in different species.

On immersion in ether, guinea-pigs yielded 0·05 to 0·1 p.c., adult rats 0·025 p.c., and rabbits 0·002 to 0·004 p.c. of their body weight in skin flakes. Mice and phalangers yielded less than 0·001 p.c. A bird, the bulbul (*Otocampea emeria*) yielded 0·005 p.c. of its body weight in skin flakes. On extracting with hot water, skin flakes lost approximately from one tenth to one half of their weight. A sample of skin flakes from bandicoots lost 38 p.c., and a sample from guinea-pigs 47 p.c. of their weight on extraction with hot water.

All aqueous skin-flake extracts contained varying amounts of nitrogenous substances which could be removed by the usual deproteinizing agents used in blood analysis. Some figures given in Tables 1 and 2 indicate the greatly varying amount of these substances, which were precipitated with tungstic acid.

After precipitation with tungstic acid, the aqueous extracts of skin flakes were found to contain all the non-keratinous substances, i.e., water-soluble organic compounds associated with horny structures which are present in the corresponding keratinous appendages. Quantitative determinations were done on total nitrogen, ammonia, urea, uric acid, amino acids before and after hydrolysis, total reducing substances before and after hydrolysis, pentose and desoxyribose.

*Total nitrogen.* In birds the total non-protein nitrogen content of the aqueous extract of skin flakes varied from 565 mg. p.c. to 1808 mg. p.c., and in the mammals it varied from 1469 mg. p.c. to 7636 mg. p.c., the highest

**TABLE 1.**

*Non-keratins in skin flakes of birds.*

| | Total N mg. p.c. | Urea N mg. p.c. | $NH_3$ N mg. p.c. | Uric acid* mg. p.c. | Amino acid N | | Reducing substances | | Pentose mg. p.c. | Polypeptide N*** mg. p.c. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | mg. p.c. | H** mg. p.c. | mg. p.c. | H** mg. p.c. | | |
| Butcher Bird | 565 | 0 | 87 | | | | 311 | 579 | 254 | |
| Pigeon | 823 | 0 | 123 | 250 | | | 869 | 1595 | 713 | |
| Pigeon barbs | 348 | 0 | 99 | 134 | | | 235 | 274 | 198 | |
| Duck 1 | 1385 | 0 | 88 | | | | | | | |
| Duck 2 | 1593 | 0 | 76 | 1420 | 134 | 187 | 2577 | 4294 | 1493 | |
| Duck 3 | 1808 | 0 | 136 | 1484 | 219 | 479 | 874 | 2264 | 884 | 45 |
| Duck barbs | 239 | 0 | 71 | 107 | | | 139 | 167 | 161 | |
| Goose | 1411 | 0 | 84 | 1227 | | | 1980 | 3805 | 1620 | |
| Goose barbs | 238 | 0 | 56 | 146 | | | | | | |

Values for the barbs of the feathers are included for comparison.
*Uric acid values were determined by the uricase method.
**Values obtained after hydrolysis.
***Nitrogen content of protein-like substance precipitated from the aqueous extract.

TABLE 2.

*Non-keratins in skin flakes of mammals.*

| | Total N mg. p.c. | Urea N mg. p.c. | $NH_3$ N mg. p.c. | Uric acid* mg. p.c. | Amino acid N | | Reducing substances | | Pentose mg. p.c. | Desoxy-ribose mg. p.c. | Polypep-tide N*** mg. p.c. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | mg. p.c. | H** mg. p.c. | mg. p.c. | H** mg. p.c. | | | |
| Koala | | 25 | 90 | | | | 860 | 1930 | 676 | | |
| Koala fur | | 32 | 70 | | | | 309 | 486 | 170 | | |
| Bandicoot | 3376 | 46 | 232 | 46 | 501 | 636 | | | 411 | | 742 |
| Bandicoot fur | 865 | | | 34 | | | | | 131 | | |
| Cat 1 | 1469 | | 99 | | | | 746 | 1813 | 488 | | |
| Cat 2 | 1593 | | | | 155 | | 724 | 2056 | | | |
| Cat 3 | 2006 | | | 115 | | | | | | | |
| Cat 4 | 2104 | | 133 | 124 | | 275 | 787 | 2651 | 462 | | 30 |
| Cat 5 | 2131 | | | | | | 880 | 4025 | 876 | | |
| Cat fur | 303 | 20 | 46 | 354 | | | 164 | 296 | 37 | | |
| Mouse | 2767 | | | | | | | | | | |
| Mouse fur | 843 | | | | | | | | | | |
| Rat | 3211 | | 272 | | | | 430 | 2053 | 480 | 108 | |
| Guinea-pig 1 | 5876 | 51 | 637 | | 513 | 513 | 1284 | 1692 | 160 | 64 | |
| Guinea-pig 2 | 6715 | 60 | 808 | | | | 609 | 678 | 112 | 158 | |
| Guinea-pig 3 | 7088 | 48 | 832 | 46 | | 646 | 1410 | 2140 | 152 | 34 | |
| Guinea-pig 4 | 7636 | 55 | 759 | 140 | 390 | 425 | 1140 | 2270 | 223 | 89 | 931 |
| Guinea-pig fur | 622 | 193 | 84 | 115 | | | 194 | 225 | 98 | 40 | |
| Rabbit 1 | 2580 | 65 | 132 | 40 | 267 | 281 | | 493 | 285 | | 765 |
| Rabbit 2 | 3390 | | | 58 | | | 501 | 967 | | | 501 |
| Rabbit fur | 516 | 86 | 36 | 584 | 25 | 40 | 213 | 446 | | | |

Values for fur are included for comparison.

* Uric acid values were determined by the uricase method.

** Values obtained after hydrolysis.

*** Nitrogen content of protein-like substance precipitated from the aqueous extract.

values being obtained from guinea-pigs. Determinations on two different breeds of rabbits, three different ducks, five non-related cats and four different groups of guinea-pigs indicate the variation in total non-keratin nitrogen which may occur within a species. The total water-soluble nitrogen content of the barbs of feathers of pigeons, ducks and geese, was only a fraction of the nitrogen content of the corresponding skin flakes. The same applied to the hair of mammals.

*Ammonia and urea nitrogen.* Ammonia nitrogen was always present in the skin flakes of birds and mammals and in the present group of animals (Tables 1 and 2) reached highest values in guinea-pigs (500 to 800 mg.).

Urea, always absent from skin flakes of birds, was always present in the skin flakes of mammals, but the quantities were smaller than those of ammonia. Furthermore, in mammals the ammonia nitrogen content rose to over 800 mg. p.c. when the total non-protein nitrogen was at the higher levels, while the urea nitrogen content remained practically constant and always below 100 mg. p.c.

The ether used for releasing the skin flakes of the cat contained urea and ammonia which, in all probability, originated from skin secretions and from saliva deposited on the fur by continual grooming. The possibility of leaching out urea from the skin flakes by ether was tested. Dry isolated cat skin flakes were ground up with about 100 volumes of ether. The amount of urea recovered in the ether was about 2 mg. per 100 gm. of skin flakes.

*Uric acid.* In birds uric acid was present from 250 to 1420 mg. p.c., and in the duck and goose uric acid supplied nearly one-third of the total nitrogen. In birds the uric acid content of skin flakes always exceeded that of the barbs of the feathers. In mammals the uric acid concentration of skin flakes was lower than in birds, and in the present group ranged from 40 to 128 mg. p.c. In the case of the cat, rabbit and the guinea-pig the fur contained from two to twelve times more uric acid than the skin flakes. In the bandicoot, which has a low fur uric-acid content, the skin-flake uric acid was of about the same order as that of the fur.

*Amino acid nitrogen.* Qualitative tests and paper chromatography indicated the presence of about nine amino acids such as leucine, valine, alanine, glutamic acid, serine, arginine, glycine, aspartic acid and threonine. Attempts were made to obtain some quantitative information on the free amino acids of the aqueous extract of skin flakes, using Folin's (1922) colorimetric method. A few determinations indicated that roughly 10 p.c. of the total non-protein nitrogen was represented by free amino acid nitrogen. After hydrolysis of the "deproteinized" aqueous extract, the amino-acid nitrogen usually increases and may become twice as high as the free amino-acid-nitrogen content.

*Reducing substances.* Reducing substances before hydrolysis ranged from 311 to 2575 mg. p.c., expressed as glucose. After hydrolysis of the aqueous skin-flake extract the reducing substances increased from 579 to 4294 mg. p.c. No definite difference could be detected between birds and mammals, and both low and

high values were present in both animal orders. Pentose and desoxyribose were always present, mainly in combined form, as shown by Tauber's reaction which became positive after hydrolysis. Pentose determinations had to be performed on the aqueous extract prior to deproteinization because the tungstic acid interfered with the orcinol method of pentose determination and the values obtained were about one-third too low. The same applied to the technique of desoxyribose determination, tungstic acid being precipitated by Dische's reagent, making colour comparison impossible. The large difference between reducing substances before and after hydrolysis can be explained partly by the presence of polysaccharides because a positive iodine reaction for polysaccharides could always be obtained. The colour produced, however, was blue, or a mixture of blue and red, and not red alone as with glycogen.

Ascending paper chromatography before and after hydrolysis revealed the presence of glucose. Furthermore, spots corresponding to those of arabinose and xylose were repeatedly obtained after hydrolysis with dilute acid, but the presence of ribose has not yet been demonstrated.

## DISCUSSION.

The epidermis proper consists almost solely of cells derived from a single type (squamous stratified epithelium), but is composed of a number of characteristic strata. The superficial layer (*stratum corneum*) is cornified and, being constantly renewed from the dividing cells in the lower layers, is shed in the form of dry amorphous skin flakes.

It has been stated (Carruthers and Suntzeff, 1953) that owing to its single cell-type composition, epidermis is rather well suited to chemical analysis. Frequently, however, it would be preferable to investigate an individual stratum. Hitherto this was considered a very difficult task, and knowledge of the biochemistry of any individual layer of the epidermis was very meagre. However, the simple methods of collecting skin flakes now described furnishes in many instances sufficient *stratum corneum* to permit an extensive analysis of their non-keratins. A definite layer of epidermal tissue can thus be obtained from a dead or living animal without interfering with the underlying epidermis. The tissue obtained is in its final static condition. Of course, these skin flakes can be harvested again and again from the same animal.

Skin flakes represent amorphous keratinous structures morphologically different from the keratinous exoskeleton which consists of definitely formed elements such as hair, nails, etc. The non-keratins of these keratinous appendages have been investigated previously (Bolliger and Gross, 1952, 1952a, 1953).

Not all birds or mammals yield conveniently large amounts of skin flakes. Sometimes, as in the phalanger or the mouse, it may be possible that skin keratinization leading to the shedding of skin flakes may be diminished if the forma-

tion of hair fibres demands much of the material offering from the epidermis. For example, the phalanger with its thick coat of fur has a thin epidermis of only 24 $\mu$ total thickness. Of this the horny layer occupies 12 $\mu$ (Bolliger and Hardy, 1944). The phalanger produces only a few skin flakes while the guinea-pig, with a thicker epidermis averaging about 44 $\mu$ on its flanks and a short coat of fur, is a prolific producer of skin flakes. The mouse, which also possesses a thin epidermis (total thickness 24 $\mu$) but a comparatively substantial coat, yields few skin flakes.

It must also be taken into consideration that some animals remove more skin flakes by preening and licking from their furs or feathers than do others which are less disposed to grooming themselves.

*Total nitrogen.* Roberts and Frankel (1949, 1949a) have examined the tissue of the whole epidermis of the mouse for total nitrogen, trichloracetic-acid-soluble-nitrogen, urea and preformed ammonia. The trichloracetic-acid-soluble-nitrogen is at present of particular interest because it is similar to the total non-keratin nitrogen obtained by hot water extraction and subsequent deproteinization. Fresh mouse epidermis was found to contain 790 mg. p.c. of trichloracetic-acid-soluble-nitrogen (Roberts and Frankel, 1949), while the total non-keratin nitrogen of mouse skin flakes was 2767 mg. p.c., i.e., about 60 p.c. of the total protein and non-protein nitrogen (4600 mg. p.c.) of the whole epidermis. In general, all skin flakes examined, with the sole exception of the half-grown butcher bird, had a much higher non-protein nitrogen content than the total epidermis of the mouse. To some degree this is to be expected since the water content of skin flakes is probably lower than that of fresh epidermis. Even when allowance is made for their water content, however, the total non-protein nitrogen content of skin flakes is very high, indicating a marked accumulation of non-protein nitrogen in the *stratum corneum*.

*Urea and ammonia nitrogen.* Homogenates of total epidermis of rat and mouse contained urea and ammonia in amounts larger than other tissues (Roberts and Frankel, 1949a). In fully grown rats the urea nitrogen content ranged from 38 to 77 mg. p.c., the ammonia nitrogen content from 26 to 45 mg. p.c. In the mouse the ranges for epidermal urea nitrogen were 55 to 98 mg. p.c., for ammonia nitrogen 16 to 31 mg. p.c.

Since each mouse yields only about 0·5 mg. skin flakes, more prolific skin-flake producers such as the bandicoot, the guinea-pig and the rabbit were chosen for urea and ammonia nitrogen determinations. In all these mammals, and also in rats and birds, larger amounts of ammonia (range 76 to 832) were found in the skin flakes than Roberts and Frankel found in the whole epidermis of the rat or the mouse. In the present series of mammals the urea-nitrogen content of skin flakes is of the same order of magnitude as in the epidermis of the mouse and the rat. Birds, however, in conformity with their uricotelic nature have no urea in their skin flakes. Furthermore, the urea nitrogen in mammalian skin flakes (range 32 to 65 mg. p.c.) is never as high as the ammonia nitrogen and is present in amounts similar to those of urea nitrogen of the whole epidermis. This indicates that, in mammals at least, ammonia accumulates in the *stratum corneum*. This ammonia may be derived by a process of deamination connected with keratinization.

*Uric acid.* The uricotelic nature of birds is strongly emphasized by the high uric-acid content of their skin flakes which frequently was about ten times that of their feathers. In mammals the situation is reversed and most of the furs examined contained larger amounts of uric acid than the skin flakes of the same animal. This is particularly marked in mammals with a very high uric-acid content in their fur such as the rabbit. This phenomenon may be

explained by the hypothesis that fur and horny layer compete for the uric acid which becomes available in the overall process of keratinization and that the fur receives the greater share.

It is apparent that urea, ammonia, amino-acid and uric-acid nitrogen account for only about one quarter to one third of total non-protein nitrogen of mammalian skin flakes and only for about half of the non-protein nitrogen of avian skin flakes. The large amount of undetermined nitrogen is being investigated particularly for purines other than uric acid.

*Reducing substances.* Expressed as glucose, one to two parts in each hundred parts of skin flakes may be reducing substances. After hydrolysis with dilute acids, the content of reducing substances may become twice as high. Among the reducing substances glucose can readily be identified while it is much more difficult to detect ribose, a substance expected to be present as a nucleic-acid degradation product. Desoxysugar, believed to be desoxyribose, could be demonstrated and measured by Dische's reaction. Desoxyribose is considered to be derived from desoxynucleic acid. The origin of arabinose and xylose, demonstrated by paper chromatography, is difficult to explain. Similarly, the blue iodine reaction for polysaccharides in skin-flake extracts of birds and mammals poses a problem, and it seems necessary to postulate the presence of a polysaccharide other than glycogen in the animal integument.

*Some aspects of comparative biochemistry.*

It has already been found that feathers and keratinous skin from the legs of birds contain no urea whatever (Bolliger and Gross, 1952a). Skin flakes from birds also contain no urea (Table 1). This confirms the statement that the presence or absence of urea in the non-keratins establishes a dividing line between birds and mammals. This fact becomes even more remarkable in view of the large amount of soluble nitrogen present in skin flakes.

Owing to the high uric-acid content of some furs (rabbit, cat, etc.), and owing to the unexplained inability of feathers to store uric acid in large amounts, i.e., over approximately 150 mg. p.c., the uricotelic trend of avian metabolism, although not obvious from comparisons of fur and feathers, becomes evident in skin flakes. In birds these show a much higher uric-acid content than mammals (Tables 1 and 2) and may contain uric-acid nitrogen up to one-third of the total non-keratin nitrogen.

The toxic nature of ammonia is well recognized and the high ammonia content of skin flakes is therefore noteworthy. The epidermis in general seems to contain considerable amounts of ammonia and Roberts and Frankel (1949a) even found small quantities of ammonia in the dermis.

*Comparison of skin flakes and keratinous appendages.*

In contrast to keratinous appendages, mammalian skin flakes contained varying amounts of water-soluble nitrogenous compounds which could be removed by the usual deproteinizing agents. At present these are considered to be polypeptides which require further study. Furthermore, mammalian fur frequently contained glycogen as shown by a red reaction with iodine while skin flakes gave mainly a blue reaction, as do birds' feathers and avian skin flakes.

In skin flakes free amino-acid nitrogen is elevated comparably with the increased total nitrogen content. After hydrolysis of the extract, amino-acid

nitrogen frequently increases, suggesting the presence of compounds of a polypeptide nature. Quantitatively, the non-keratins of shed snake skin (Bolliger and Gross, 1952) are more closely related to those of skin flakes of birds than to feathers. However, the amounts of the substances determined in snake skin are, on an average, still only about half or less of the corresponding amounts for avian skin flakes. Thus it appears that, quantitatively, the non-keratins of snake skin lie somewhere between those existing in the keratinous exoskeleton, i.e., the feathers and the keratinous skin layers of birds.

Compared with the roots of feathers and hair, which possess a vascular system which may transport non-keratins to or from other tissues, the skin flakes, and in fact the whole keratinous layer, have no such connection and the non-keratins are forced to reside where they are produced. Thus skin-flake non-keratins may give a more quantitative picture of the process of keratinization than do the keratinous appendages themselves.

The presence of water-soluble proteins, polypeptides and large amounts of free amino acids in skin flakes could be considered an indication of a decomposition process of keratin. This was considered to be unlikely on account of the absence of any cystine in the aqueous extract (Bolliger and Gross, 1954).

The non-keratins in skin flakes and keratinous appendages are the by-products of epidermal catabolism which leads to keratin as its main product. Chemically speaking, nucleic-acid degradation is probably the most impressive phase of this process, but the possible importance of ammonia, free amino acids and mono and polysaccharides must not be overlooked.

The rapid renewal of the skin surface layer of the epidermis and the consequent large production of desquamatory products containing large amounts of non-keratin is in all probability necessary for protective purposes and for speedy repair after injury.

## SUMMARY.

A simple method for the collection of cast off *stratum corneum*, referred to as skin flakes, is described. This hitherto neglected tissue was examined in birds and mammals for its content of non-keratins, i.e., water-soluble organic compounds.

As much as one-third or more of this cornified tissue was found to consist of water-extractable organic compounds including varying amounts of a water-soluble protein-like substance.

All of the non-keratinous compounds previously identified in keratinous appendages were found to be present in skin flakes. Total non-protein nitrogen, ammonia, urea, uric acid, amino acids before and after hydrolysis, reducing substances before and after hydrolysis, pentose and desoxyribose were determined quantitatively.

Total non-protein nitrogen content was several times larger in skin flakes (in guinea-pigs, 8 to 12 times) than in the corresponding keratinous appendages, and the remarkably high ammonia-nitrogen fraction of skin flakes was always in excess of that of urea, which constituent was completely absent in birds.

In accordance with the uricotelic nature of birds, uric acid was much more prominent in avian than in mammalian skin flakes. In birds urates represent the main nitrogenous component.

Free amino acids, the second largest known source of nitrogen in skin flakes of birds and mammals, increase after hydrolysis of the skin-flake extract. This may indicate the presence of combined amino acids, probably in the form of a polypeptide compound. Partition chromatography revealed at least nine amino acids.

A large proportion of the non-keratins possess reducing properties which increase after hydrolysis. Of these, glucose, and on hydrolysis, arabinose and xylose, were identified chromatographically. The presence of polysaccharides other than glycogen was suggested.

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# THE EFFECT OF GLUCOSE ON BLOOD PYRUVATE IN SHEEP

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A correlation exists between the concentration in the blood of total reducing sugar and of pyruvate, an important intermediary in carbohydrate metabolism. Flock, Bollman and Mann (1938) reported that the levels of pyruvate in the blood of dogs were approximately doubled after the injection of a solution of glucose, but no rise occurred after injection of physiological saline. Bueding, Stein and Wortis (1941) observed a temporary rise in blood pyruvate after the administration of glucose to humans and dogs; in a subsequent paper, Bueding, Wortis and Fein (1942) showed that the ingestion of glucose by human diabetics brought about little increase in blood pyruvate, but if insulin was administered a significant rise occurred under these conditions. Horwitt, Hills and Kreisler (1949) observed a sharp rise in blood pyruvate after the ingestion of glucose by normal human subjects, and that the return to normal was parallel to the blood sugar curve. When glucose is injected intravenously the blood pyruvate remains elevated for approximately 2 hours (Amatuzio *et al.*, 1952).

In view of the nature of the intermediary metabolism of the adult ruminant (Marston, 1939; McCandless and Dye, 1950; Jarrett and Potter, 1953), and of the apparent similarity in some respects of a young lamb and a non-ruminant (Jarrett and Potter, 1952), the effect of an injection of glucose on the blood pyruvate level in the young lamb and the adult sheep was compared.

## METHODS.

Five adult Merino ewes that had been sustained on an adequate diet of a wheaten hay and lucerne chaff mixture were fasted overnight prior to each experiment. A small-bore polyethylene tube was inserted for several inches into the jugular vein about an hour preceding the experimental period and filled with a dilute solution of heparin in physiological saline to reduce the possibility of blood clots forming in the tube. Blood samples were obtained with a minimum of handling by attaching a syringe to the end of the tube. This procedure allowed the animal to remain placid throughout the manipulations, a condition which is essential for such experiments as these, because activity influences the concentration of pyruvate in the blood.

An initial blood sample was taken, and then 100 ml. of glucose solution, containing 1 gm. glucose/kg. body weight were injected slowly over a period of five minutes. Blood samples were taken at intervals of 30, 60 and 90 minutes after the injection, and the concentration of pyruvate determined by the method of Friedemann and Haugen (1943).

Seven Merino lambs aged 3–4 weeks were removed from their dams 2–3 hours before receiving an intravenous injection of 20 ml. of glucose solution (1 gm. glucose/kg.), and blood samples were taken at intervals under the same conditions.

Although handling of the animals was reduced to a minimum, the possibility that excitement might contribute to the alterations in the pyruvate level was assessed by an injection of physiological saline. The differences between these levels after saline and those after glucose were analysed statistically to determine the real effect of glucose on the pyruvate level in the blood.

In order to reduce further any possible effects of conscious reaction, this procedure was repeated with each of these animals under anaesthesia induced with intravenous Nembutal. The anaesthesia was established 30 minutes prior to taking the initial blood sample, and it was maintained for the duration of the experiment.

Four rabbits were subjected to the same treatment as the ruminants, except that the effect under anaesthesia was not determined; the blood samples were obtained from a marginal ear vein.

## RESULTS.

The experiments were of such a nature that many possible interactions could be involved and an analysis of variance was therefore performed.

Injection of glucose into the rabbits led to a significant rise in the level of pyruvate in the blood compared with that following saline. The maximum difference occurred 30 minutes after injection, and thereafter the concentration of pyruvate fell to a value nearer normal, so that at 90 minutes after injection no difference was apparent. The average initial sample was 1·36 mg. per 100 ml., and the mean difference between glucose and saline at 30 minutes was +0·78 mg. per 100 ml.

The increase was less evident in the young lambs, but the difference between the concentration of pyruvate after glucose and after saline was sufficiently great to be significant (cf. the interaction in Table 2). As with rabbits, the difference was most pronounced 30 minutes after injection, and by the end of 90 minutes the value was usually below that of the initial sample.

After the injection of glucose the blood pyruvate in adult sheep, however, rose steadily, and a pronounced difference between the glucose and saline effect was still evident at the end of the experimental period.

The effect of anaesthesia was more pronounced in the lambs than in the adult sheep. The initial sample in the lambs was of the same order whether taken under normal conditions or during anaesthesia, but subsequent samples were all lower in the anaesthetized animals. This was true for those animals injected with glucose as well as for those injected with saline.

In the adults under anaesthesia there was still a significant difference in the level of pyruvate after glucose and saline at the end of the 90-minute period.

Details of the experiments are set out in Table 1, and a summary of the results based on the statistical analysis is given in Table 2.

TABLE 1.

| | Normal | | | | | | | | Anaesthetized | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | | | | Saline | | | | Glucose | | | | Saline | | | |
| Time (mins.) | 0 | 30 | 60 | 90 | 0 | 30 | 60 | 90 | 0 | 30 | 60 | 90 | 0 | 30 | 60 | 90 |
| Sheep | 0·68 | 0·84 | 0·92 | 0·84 | 0·72 | 0·66 | 0·63 | 0·64 | 0·90 | 0·83 | 0·82 | 0·94 | 0·66 | 0·60 | 0·61 | 0·50 |
| | | | | | S.D. of the mean of 5 observations 0·063 | | | | | | | | | | | |
| Lambs | 1·35 | 1·39 | 1·25 | 1·19 | 1·59 | 1·33 | 1·34 | 1·38 | 1·35 | 1·06 | 0·88 | 0·71 | 1·41 | 0·96 | 0·79 | 0·96 |
| | | | | | S.D. of the mean of 7 observations 0·075 | | | | | | | | | | | |
| Rabbits | 1·27 | 1·80 | 1·42 | 1·49 | 1·46 | 1·02 | 0·88 | 1·49 | | | | | | | | |
| | | | | | S.D. of the mean of 4 observations 0·154 | | | | | | | | | | | |

TABLE 2.

| | Sheep | Lambs | Rabbits |
|---|---|---|---|
| Glucose v. saline | Glucose>saline (·001 level). (Glucose v. saline)×(anaesthetized v. normal) interaction significant (at ·05 level). This is due to glucose v. saline difference being significantly greater in anaesthetized sheep than in normal sheep. | Not significant. In initial and 90 mins. Glucose is significantly (·01 level) lower than saline. In 30 and 60 mins. glucose and saline are not significantly different. | Significant (at ·05 level). Saline is significantly lower (at ·05 level) than glucose. But there is a treatment×times interaction, the response to glucose at 30 and 60 minutes being significantly greater than the response at 0 and 90 minutes. |
| Anaesthetized v. normal | Not significant. | Anaesthetized significantly lower than normal (·001 level). Initially anaesthetized is not significantly different from normal; at other times it is significantly lower (at ·001 level). | |
| Times | Not significant. | Significant (at ·001 level). Times are significant in both glucose and saline. Times are not significantly different in normal but are significantly (at ·001 level) different in anaesthetized. | Not significant. But in glucose or in saline, considered separately times are significant (at ·05 level). |
| Animals | Not significant. | Significant (at ·01 level). Animal × (glucose v. saline) interaction is significant (at ·05 level). All animals differ in (glucose v. saline) response. | Not significant. Animal×treats. Interaction significant (at ·05 level). All animals differ in treatment response. This makes the over-all treatment effect doubtful, but not the difference at 30 and 60 minutes. |
| (Glucose v. saline) ×times interaction | Not significant. But the regression of (glucose-saline) differences on Times; (in which a straight line gives a good fit) was significant at ·05 level. | Significant (at ·05 level). This is due to the different response of glucose and saline to time. Considered separately there is no significant effect in normal or anaesthetized. | Significant (at ·05 level). Due to different response of glucose and saline to time. |
| (Anaesthetized v. normal) ×times interaction | Not significant. | Significant (at ·01 level). This is due to a differential response in anaesthetized and normal animals at the several times. | |

## DISCUSSION.

The concentration of pyruvate in the blood of normal rabbits was found to agree closely with that reported previously (Lu, 1939), but the normal value for adult sheep was found to be much lower than that observed by Austin (1947). However, this difference might well be attributed to the different experimental conditions, as it is clear that a state of activity affects the pyruvate concentration in the blood: to obtain reasonably comparable results it is essential that the animal be maintained in a thoroughly placid condition (Jarrett, Potter and Filsell, 1952). The technique of intravenous cannulation used in this study assisted considerably towards this end.

The fairly consistent results obtained with the adult sheep reflected their placidity: it was much more difficult to reduce the activity of the lambs. When they were removed from the ewe they became excited, and even though handling was reduced to a minimum, the results obtained were variable. The primary purpose of anaesthesia was to effect some degree of control so that a minimum initial value could be obtained. The gradual decrease in blood pyruvate which occurred during the period of anaesthesia indicated that, in the lambs, activity may have been a complicating factor. However, when the differences between the response to glucose and saline were determined, the values obtained were sufficiently great to be significant.

The rise in blood pyruvate which occurred in the rabbits following glucose injection agreed with the results reported for dogs (Flock, Bollman and Mann, 1938) and for humans (Bueding, Stein and Wortis, 1941; Amatuzio *et al.*, 1952). In the blood of adult sheep, however, the level of pyruvate gradually increased and after 90 minutes was still higher than the normal value: the magnitude of this rise was, however, considerably less than that observed in rabbits.

The rise in blood pyruvate in adult sheep was much less after glucose than that observed after the injection of a comparable amount of sodium propionate (5 mM/kg.) which resulted in an average rise of 4 mg./100 ml. No alteration followed an injection of sodium acetate (Jarrett, Potter and Filsell, 1952).

Another interesting feature is that the rise in pyruvate level followed the trend of the glucose tolerance curve, a phenomenon previously reported for both normal and diabetic humans by Horwitt, Hills and Kreisler (1949). The glucose tolerance curve of the young lamb is similar to that of non-ruminants in that it returns to normal after a period of 1½ to 2 hours: that of the adult sheep, however, is more prolonged and may be compared with the curve for diabetic non-ruminants in that the return to normal is delayed for 3 to 4 hours.

This phenomenon may be associated with a relative inability of the tissues of the adult ruminant to utilize glucose as suggested by Reid (1952). The effect of propionate, which brings about a greater rise in blood pyruvate than glucose, might then be explained by a more direct conversion of the propionate to pyru-

vate. The reports of Bueding, Wortis and Fein (1942) and of Horwitt, Hills and Kreisler (1949) that a small and delayed rise occurred in diabetic humans may be significant in this respect in view of the metabolic blocks that occur in diabetes (Chaikoff, 1952).

If the theory that diabetes is due to an impaired utilization of glucose is accepted, the reactions of a normal adult sheep to a glucose injection seem to be comparable with that of a diabetic non-ruminant.

## SUMMARY.

The concentration of pyruvate in the blood of sheep, lambs and rabbits was estimated after an intravenous injection of glucose.

The reactions of the young lamb were like those of the rabbit: the response was an elevated blood pyruvate which had returned to normal by the end of 90 minutes.

The adult sheep, however, responded differently: the pyruvate level, like that of the blood sugar level, remained elevated 90 minutes after the injection.

The possible explanation of this phenomenon in the adult ruminant is discussed.

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# THE INTERRELATIONSHIPS OF THE PLASMA AND LYMPH LIPIDE FRACTIONS BEFORE AND DURING FAT ABSORPTION

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The changes in the thoracic duct lymph lipide picture that occur with fat absorption are well recognized, but the significance of the interrelationships of these lipide fractions and their relative importance in the absorption and transport of ingested fat is still debatable. The possibility that phospholipide, neutral fat, cholesterol and its esters vary in the animal organism in a closely related and complementary manner has led to a study of the lipide interrelationships that may exist in different physiological and pathological states. This present investigation is an attempt to evaluate the significance of the lipide fractions of the lymph and plasma before and during fat absorption. The plasma-lymph lipide equilibrium has been studied to assess the extent to which the plasma lipides are involved in the extravascular space-lymphatic circulation which characterizes the plasma proteins and to determine the permeability of the capillary membrane to lipide molecules.

## METHODS.

*Animals.* Cats, rabbits and rats were used for these experiments. The cats were of mixed sexes with weights varying between 2 and 5 kg. The rabbits were hutch-bred albino and Himalayan males, weighing between 1·5 and 3·0 kg. The rats were an albino Wistar strain, 180–250 gm. in weight.

*Anaesthesia.* Cats and rabbits were anaesthetized with nembutal (Abbott) given intravenously, anaesthesia being maintained in some cases by ether inhalation. Rats were given ether and allowed to recover from the anaesthetic on completion of the operation.

*Lymphatic cannulation.* In cats and rabbits the thoracic duct was cannulated near the jugular venous confluence at the base of the neck, using a glass or polythene cannula. All visible communicating branches were tied off and the lymph flows recorded were spontaneous. In rats, the thoracic duct was cannulated in the abdomen using "Transflex" plastic tubing (1¼ mm. external diameter). The technique adopted was that described by Bollman, Cain and Grindlay (1948). After completion of the operation, the rat was placed in a restraining cage for the duration of the experimental period. The lymph was collected from the cannulae into heparinized, graduated, centrifuge tubes.

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*Blood samples.* In cats and rabbits, the samples were obtained from the femoral artery through polythene catheters. A polythene catheter was inserted through the saphenous vein into the inferior vena cava for the administration of substances intravenously. In rats, terminal blood samples were obtained by direct cardiac puncture. Dry heparin was used as an anticoagulant.

*Lymph transfusion.* Homologous thoracic duct lymph was obtained from fat fed animals for subsequent transfusions. The lymph was stored overnight in the refrigerator and given intravenously (40 ml./kg. body weight) as a constant infusion lasting about ½ to ¾ of an hour.

*Protein determinations.* Total protein and N.P.N. estimations were carried out on plasma and lymph by microkjeldahl digestion and direct nesslerization. Optical densities were measured on a Hilger Spekker absorptiometer.

*Total fatty acid.* The Man and Gildea (1932) modification of the Stoddart and Drury titrimetric method was used for the most part. In the later series of estimates on rabbit and rat plasma and lymph, the colorimetric technique described by Stern and Shapiro (1953) was used. Both these methods record the combining power of the fatty acid present, so concentrations are given in milliequivalents/litre.

*Phospholipide.* The lipide phosphorus determinations were made on plasma and lymph using a micro modification of the method of King (1932). Optical densities of the solutions were recorded on a Beckman spectrophotometer at a wavelength of 830 mμ, 30 minutes after colour development. The factor of 25 was used to convert lipide phosphorus to lecithin.

*Total cholesterol.* The technique of direct chloroform extraction, described by Kingsley and Schaffert (1949), was used. Colour development was achieved with concentrated sulphuric acid and acetic anhydride reagent and the optical densities read at a wavelength of 620 mμ.

*Neutral fat.* This was calculated by difference according to the method of Peters and Man (1943) as total fatty acid minus (cholesterol fatty acid plus phospholipide fatty acid). Cholesterol was taken as existing wholly in the esterified form whilst all phospholipide was considered as lecithin. These assumptions are recognized sources of error in this estimate.

*Plasma volume estimates.* Plasma volumes of 35 ml./kg. for cats, 38 ml./kg. for rabbits (Korner, Morris and Courtice, 1954) and 36 ml./kg. for rats (Montgomery, 1951) were used in calculating the total circulating phospholipide.

*Dietary states.* Cats were divided into two groups. A post absorptive group was kept without food for 24 hours before the experiment. Thoracic duct lymph and plasma samples were taken and a fat meal (olive oil, 3 ml./kg.) was then given by stomach tube. A second group was fed a fat meal prior to the experiment and, when absorption was well advanced, the thoracic duct was cannulated and plasma and lymph samples obtained.

Rabbits were divided into three groups. One group was fed for a period of one month before the experiment on a low fat diet of vegetable leaves. The second group was fed on rabbit cubes (Animal Health Products, 4 p.c. fat), whilst the third group was given milk (4 p.c. fat). All received water *ad lib.* Olive oil (3 ml./kg.) was given by stomach tube for the fat meal.

Rats were fed on rat nuts (Animal Health Products, 2·4 p.c. fat) and given a fat meal (1 ml. olive oil) through a gastrostomy tube. The gastrostomy was established by the insertion of a small piece of rubber tubing with polythene attached into the stomach near the cardia. The tube was held in place by a purse string suture and exteriorized through a stab wound in the dorsal wall of the abdomen.

TABLE 1.

*The protein and lipide content of the plasma, thoracic duct and cervical duct lymph of post-absorptive cats.*

| | Plasma | | | | Thoracic duct lymph | | | | Cervical duct lymph | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Phospholipide | | | | Phospholipide | | | | Phospholipide |
| Cat. No. | Total protein gm. p.c. | Total fatty acid m.eq./l. | Total cholesterol mg. p.c. | phos-phorus mg. p.c. | Total protein gm. p.c. | Total fatty acid m.eq./l. | Total cholesterol mg. p.c. | phos-phorus mg. p.c. | Total protein gm. p.c. | Total fatty acid m.eq./l. | Total cholesterol mg. p.c. | phos-phorus mg. p.c. |
| 1 | 7·5 | 13·0 | 118 | 6·51 | 4·7 | 11·0 | 45 | 4·95 | 3·7 | 6·8 | 21 | 4·47 |
| 2 | 6·6 | 8·8 | 75 | 6·54 | 3·8 | 5·3 | 31 | 4·56 | 2·0 | 3·5 | 27 | 4·62 |
| 3 | 6·3 | 11·0 | 72 | 5·40 | 3·9 | 7·1 | 29 | 2·20 | 2·9 | 2·2 | 16 | 1·60 |
| Mean | 6·8 | 10·9 | 88 | 6·15 | 4·1 | 7·8 | 38 | 3·90 | 2·9 | 4·2 | 21 | 3·56 |

## RESULTS.

### Experiments With Post-absorptive Animals.

*Protein and lipide content of the plasma, thoracic duct and cervical duct lymph.*

The protein and lipide content of the plasma, thoracic duct and cervical duct lymph of cats in the post-absorptive state is given in Table 1. As with protein, the cervical duct lymph contains the smallest amount of lipide and there exists a concentration gradient between the plasma and the lymph. If it is assumed that in the post-absorptive state the lymph lipides have origin in their plasma counterparts, this concentration gradient expresses the extent to which these substances diffuse through the capillary membrane into the extravascular space. The concentrations of total fatty acid, total cholesterol and phospholipide in the thoracic duct lymph of a series of 15 post-absorptive cats were found to be, respectively, 73, 47 and 54 p.c. of the plasma levels (Table 2). These lipide fractions in the plasma of the post-absorptive animal are thought to be associated principally with the $\alpha$-globulins, and, consequently they would be expected to have a similar plasma-lymph equilibrium gradient. It has not been possible to determine the concentrations of $\alpha$-globulins in the plasma and lymph, but in the cat, the electrophoretic patterns of plasma and lymph are similar and the total globulin concentration in the lymph is on an average 60 p.c. of that of the plasma (Korner, Morris and Courtice, 1954). The plasma-thoracic duct lymph-lipide equilibria are expressed by regression equations derived from estimates of the concentrations of the fractions in both fluids in Table 2.

TABLE 2.

*The regression equations for the thoracic duct lymph and plasma lipide fractions in the post-absorptive cat.*

| | No. of results | Regression equation | Regression coefficient | Significance |
|---|---|---|---|---|
| Total fatty acid | 15 | T.D.T.F.A.=7·13+0·680 (P.T.F.A.−9·73) | 0·680±0·257 | 0·02–0·01 |
| Neutral fat | 15 | T.D.N.F.=5·38+0·829 (P.N.F.−4·82) | 0·829±0·316 | 0·02–0·01 |
| Phospholipide phosphorus | 18 | T.D.P.P.=3·79+0·649 (P.P.P.−7·02) | 0·649±0·215 | 0·01 |
| Total cholesterol | 19 | T.D.T.C.=46·21+0·328 (P.T.C.−99·32) | 0·328±0·090 | 0·001 |

T.D.T.F.A.=Thoracic duct total fatty acid concentration, m.eq./l.
T.D.N.F.=Thoracic duct neutral fat concentration, m.eq./l.
T.D.P.P.=Thoracic duct phospholipide phosphorus concentrations, mg. p.c.
T.D.T.C.=Thoracic duct total cholesterol concentration, mg. p.c.
P.T.F.A.=Plasma total fatty acid concentration, m.eq./l.
P.N.F.=Plasma neutral fat concentration, m.eq./l.
P.P.P.=Plasma phospholipide phosphorus concentration, mg. p.c.
P.T.C.=Plasma total cholesterol concentration, mg. p.c.

*The interrelationships of the lipides in plasma and lymph.*

To assess the significance of any interrelationships existing between the fat fractions of the plasma, a multiple regression analysis of total cholesterol and phospholipide phosphorus on total fatty acid was carried out on a series of 19 estimates in the post-absorptive cat. The concentration of total fatty acid present in the plasma was found to be directly related to the plasma phospholipide phosphorus and total cholesterol content. This relationship is represented graphically in Fig. 1 (a), where cholesterol changes are represented along the line $X_1$ and phospholipide phosphorus along the line $X_2$. A similar analysis was carried out on a series of 20 thoracic duct lymph determinations. The concentration of total fatty acid present in the lymph was found to be very significant correlated to the amount of lymph phospholipide, but in this series of results the cholesterol fraction was non-significant. This relationship is shown in Fig. 1 (b). It is apparent that in both the plasma and the lymph, the phos-

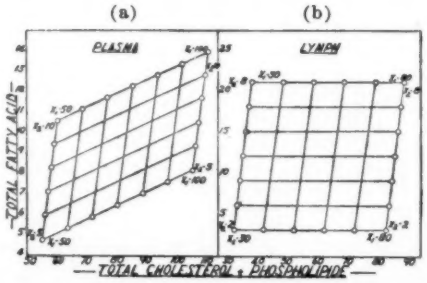


**Fig. 1.** The effect of changes in the total cholesterol and phospholipide phosphorus content on the concentration of total fatty acid in the plasma and thoracic duct lymph of post-absorptive cats.

$X_2$=Phospholipide phosphorus (mg. p.c.).
$X_1$=Total cholesterol (mg. p.c.).

The ordinate shows the total fatty acid concentration in milliequivalents/litre.

pholipide fraction plays a very significant part in influencing the concentration of total fatty acid present. The regression equations from which Fig. 1 is derived are given in Table 3.

*The removal of transfused lipides from the circulation.*

To establish the rate at which fatty material is removed from the circulation, post-absorptive cats were transfused with homologous lymph of high fat content. The rate of removal was found to be quite rapid and plasma levels returned to normal in about 2–3 hours after the transfusion ceased. To determine whether the removal of lipide from the circulating plasma involved filtration into the extravascular space and subsequent collection by the lymphatics, the thoracic

TABLE 3.

*The regression equations of phospholipide phosphorus and total cholesterol on total fatty acid in the plasma and thoracic acid duct lymph of cats.*

| Experiment | No. of results | Multiple regression equation | First regression coefficient | Significance | Second regression coefficient | Significance |
|---|---|---|---|---|---|---|
| Post-absorptive plasma | 19 | P.T.F.A.=10·7+0·068 (P.T.C.−97·1) +1·156 (P.P.P.−7·34) | 0·068±0·024 | 0·05–0·01 | 1·156±0·58 | 0·05 |
| Post-absorptive lymph | 20 | T.D.T.F.A.=9·1+0·065 (T.D.T.C.−43·6) +3·042 (T.D.P.P.−4·0) | 0·065±0·063 | Not significant | 3·042±0·67 | <0·001 |
| Fat-fed lymph | 41 | T.D.T.F.A.=80·9+0·380 (T.D.T.C.−89·9) +5·023 (T.D.P.P.−14·3) | 0·379±0·153 | 0·02 | 5·023±0·55 | <0·001 |

P.T.F.A.=Plasma total fatty acid concentration, m.eq./l.
P.T.C.=Plasma total cholesterol concentration, mg. p.c.
P.P.P.=Plasma phospholipide phosphorus concentration, mg. p.c.
T.D.T.F.A.=Thoracic duct lymph total fatty acid concentration, m.eq./l.
T.D.T.C.=Thoracic duct lymph total cholesterol concentration, mg. p.c.
T.D.P.P.=Thoracic duct lymph phospholipide phosphorus concentration, mg. p.c.

duct lymph lipide levels were estimated simultaneously. After a lag period of about half an hour, the thoracic duct lymph showed an increase in lipide content, and plasma-lymph equilibrium was reached 2–3 hours after transfusion ceased. The plasma and thoracic duct lymph lipide levels at various time intervals following the transfusion of fatty lymph in the cat are shown in Fig. 2. In the experiment depicted in Fig. 2 the transfusion lymph had the following lipide

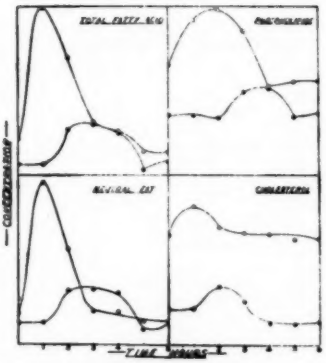

**Fig. 2.** The removal of transfused lipides from the circulating plasma of the post-absorptive cat and its appearance in the thoracic duct lymph following the intravenous infusion of lipaemic lymph (40 ml./kg.).
○=Plasma. ●=Thoracic duct lymph.

concentrations: Total fatty acid 94·3 m.eq./l., phospholipide 175 mg. p.c., and total cholesterol 170 mg. p.c. At the end of the transfusion, the plasma total fatty acid had risen from 16·1 to 59·4 m.eq./l., the phospholipide from 130 mg. p.c. to 182 mg. p.c., and the total cholesterol from 191 mg. p.c. to 243 mg. p.c. The thoracic duct lymph showed the following lipide increases: Total fatty acid 8·1 to 19·5 m.eq./l., phospholipide 70 to 103 mg. p.c., total cholesterol 69 to 101 mg. p.c., and neutral fat 6·5 to 16·1 m.eq./l.

It is evident from these results that at least some of the transfused lipide filtered through the capillary membrane into the extravascular space and was involved in an extravascular space-lymphatic circulation. The rapid rate at which fat is removed from the circulation makes the investigation of the lipide picture in fat absorption by means of blood fat analysis more difficult to interpret. For this reason lipide analysis during fat absorption was confined mainly to the thoracic duct lymph.

### Experiments During Fat Absorption.

***The lymph lipides.***

Having investigated the lipide interrelationships in the post-absorptive animal, an attempt was made to assess the significance of the different lipide

fractions during fat absorption. The pure fat, olive oil, was used to eliminate any exogenous cholesterol or phospholipide. The thoracic duct lymph flow and lipide levels during a period of 10 hours following a meal of olive oil in the cat, rabbit and rat is shown in Fig. 3.

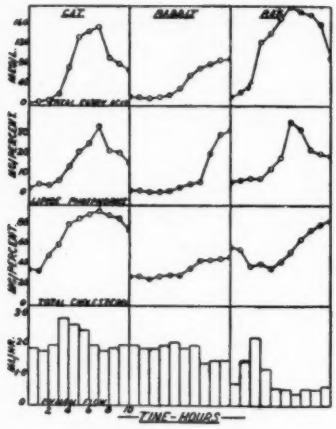


**Fig. 3.** The thoracic duct lymph lipide picture and lymph flow patterns following the feeding of olive oil in the cat, rabbit and rat.

In the anaesthetized cat, a lag period of 2–4 hours was followed by a rapid increase in the lipide content of the lymph. There was, in all cats examined, a concomitant increase in the total fatty acid, neutral fat, total cholesterol and phospholipide. The times of increase followed each other closely, but the degree of increase of each fraction varied greatly. The increases in total fatty acid and neutral fat were of the order of 10 times the post-absorptive level, the phospholipide some 5 times and the total cholesterol 2–3 times. The time of maximum fat content in the lymph varied from 4–8 hours after giving the fat meal.

In the anaesthetized rabbit, fat absorption was much slower but, nevertheless, qualitatively the same. All fractions followed one another closely, but did not reach the high levels seen in the cat. In the unanaesthetized rat evidence of fat absorption was apparent after 1 hour had elapsed. The lymph became quite milky and the total fatty acid and phospholipide phosphorus levels rose strikingly. The total cholesterol concentration, however, showed a fall, but this fall was not reflected in the hourly output of total cholesterol by the thoracic duct lymph. The rat showed a consistent increase in lymph flow, occurring synchronously with the beginning of fat absorption. This increase in the rate of lymph flow was of the order of 100–500 p.c. over the resting level and served to dilute the lymph and mask an actual increase in cholesterol output by the

thoracic duct. This increase in lymph flow was also seen consistently in the anaesthetized cat, but was less in degree and maintained for a shorter period.

The lipide concentrations of the plasma and thoracic duct lymph of the cat, rabbit and rat under different dietary conditions are shown in Table 4. The rabbit and rat had significantly lower levels of lipide in both the plasma and the lymph than did the cat. Rabbits fed on a 4 p.c. fat diet showed increases in blood and lymph fat levels over those maintained on a low fat diet of vege-

TABLE 4.

*Plasma and lymph lipide levels in the cat, rabbit and rat.*

CATS

| Group | Sample | Total fatty acid m.eq./l. | Total cholesterol mg. p.c. | Phospholipide phosphorus mg. p.c. |
|---|---|---|---|---|
| Post-absorptive | Plasma | 9·7±0·9 | 99·3±8·7 | 7·0±0·26 |
| | Thoracic duct lymph | 7·1±1·03 | 46·2±4·3 | 3·8±0·28 |
| Absorptive | Plasma | 12·9±2·36 | 110·1±7·2 | 8·1±0·17 |
| | Thoracic duct lymph | 102·7±40·9 | 93·3±5·1 | 12·8±2·45 |

RABBITS

| Group | Sample | Total fatty acid m.eq./l. | Total cholesterol mg. p.c. | Phospholipide phosphorus mg. p.c. |
|---|---|---|---|---|
| Low fat diet | Plasma | 6·3±0·95 | 28·0±1·8 | 2·1±0·17 |
| | Thoracic duct lymph | 7·7±0·78 | 27·0±4·2 | 2·4±0·04 |
| 4 p.c. fat diet: Rabbit cubes | Plasma | 7·7±1·9 | 37·3±5·2 | 3·6±0·38 |
| | Thoracic duct lymph | 14·6±3·2 | 49·7±2·3 | 3·8±0·52 |
| 4 p.c. fat diet: Milk | Plasma | 13·9±2·0 | 54·6±21·9 | 5·1±0·51 |
| | Thoracic duct lymph | 17·0±1·8 | 43·7±6·1 | 5·5±0·1 |

RATS

| Group | Sample | Total fatty acid m.eq./l. | Total cholesterol mg. p.c. | Phospholipide phosphorus mg. p.c. |
|---|---|---|---|---|
| Rat nuts: 2·4 p.c. fat | Plasma | 7·2±0·5 | 52·0±11·8 | 5·2±0·91 |
| | Thoracic duct lymph | 41·8±8·2 | 77·0±12·1 | 11·7±4·27 |

table leaves. All three lipides were elevated, but the plasma total cholesterol and phospholipide levels were still only about half those found in the plasma of the post-absorptive cat. In the rat, the plasma lipide levels were higher than those of the rabbit, but significantly lower than those found in the cat. The lipide levels were higher in the lymph than in the plasma in all three species, when active absorption was proceeding.

*Lymph lipide interrelationships.*

The relationship of the phospholipide and total cholesterol content of the thoracic duct lymph to the total fatty acid content during fat absorption in the cat is shown graphically in Fig. 4. The changes in phospholipide phosphorus

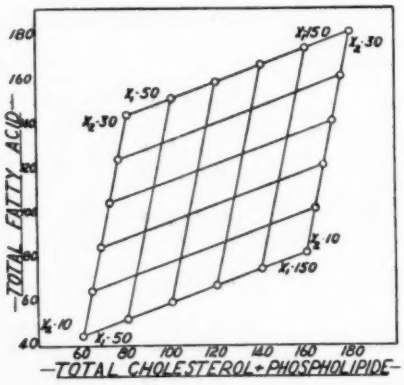


**Fig. 4.** The interrelationships of the lipide fractions of the thoracic duct lymph during fat absorption. Changes in the total cholesterol and phospholipide content of the lymph are shown along the lines $X_1$ and $X_2$ respectively.

concentration, represented along the line $X_2$, are responsible for a much greater effect on the total fatty acid content of the lymph than are changes in the concentration of total cholesterol, shown along the line $X_1$.

To evaluate the relative significance of these lipide interrelationships during fat absorption, the ratio of phospholipide phosphorus to total fatty acid was plotted against the total fatty acid content of the thoracic duct lymph before and during the absorption of a fat meal. The results for three cats are shown in Fig. 5, together with the corresponding regression lines for the logarithmic transformations of the same series of estimates. An inverse relationship is seen to exist between the regression of this ratio on the total fatty acid concentration of the lymph. As the fat starts to appear in the lymph early in the absorption phase, there is a very rapid drop in the phospholipide/total fatty acid ratio. This decline continues at a slower rate as the total fatty content reaches high levels

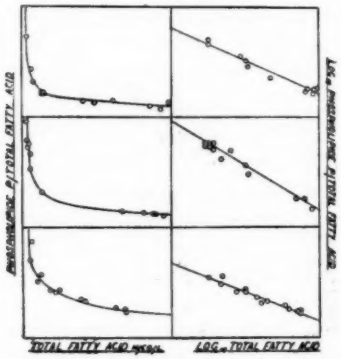

**Fig. 5.** The relationship of total fatty acid to phospholipide phosphorus in the thoracic duct lymph of cats during fat absorption. Results are shown for three individual animals.

in the lymph. The phospholipide increase has been shown to bear a highly significant relationship to the total fatty acid content of the lymph, but it is obvious that the rate of increase of lipide phosphorus is not proportional to the increase in total fatty acid. The equations of the regression lines in Fig. 5 are given:

(1) Log. P./T.F.A.=0·444—0·465 (log. T.F.A.—1·654) P.=<0·001.

(2) Log. P./T.F.A.=0·700—0·597 (log. T.F.A.—1·127) P.=<0·001.

(3) Log. P./T.F.A.=0·511—0·414 (log. T.F.A.—1·268) P.=<0·001.

where P.=lymph phospholipide phosphorus; T.F.A.=lymph total fatty acid. In the unanaesthetized rat, a similar relationship was found to exist. The fall in lymph phospholipide/total fatty acid ratio occurring during fat absorption was associated with the appearance of large amounts of neutral fat, and only about 10 p.c. of the total fatty acids present in the thoracic duct lymph was combined as phospholipide.

*Interrelationships of lipides in plasma and lymph.*

In a series of post-absorptive animals the thoracic duct was cannulated and samples of lymph and plasma collected. Olive oil, 3 ml./kg., was then given by stomach tube and when fat absorption was proceeding, lymph and plasma samples were again collected. The plasma phospholipides did not alter significantly under these conditions. The regression of thoracic duct phospholipide phosphorus on plasma phospholipide phosphorus was derived from results taken before the fat meal and is given by the equation:

T.D.P.P.=3·79+0·549 (P.P.P.—7·02). P.=0·001.

where T.D.P.P.=thoracic duct phospholipide phosphorus.

P.P.P.=plasma phospholipide phosphorus.

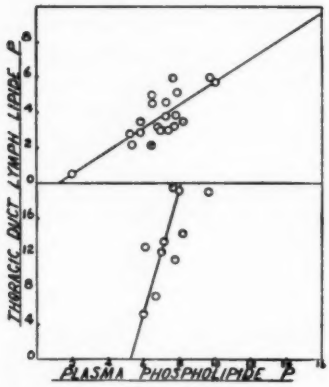


**Fig. 6.** The relationship of plasma phospholipide phosphorus (mg. p.c.) to thoracic duct lymph phospholipide phosphorus (mg. p.c.) in the cat.
Above: Post-absorptive. Below: During fat absorption.

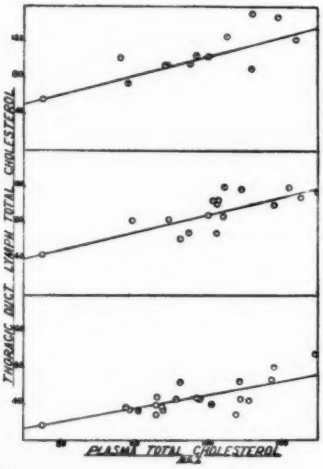


**Fig. 7.** The relationship of plasma total cholesterol (mg. p.c.) to thoracic duct lymph total cholesterol (mg. p.c.) in the cat.
Above: Groups of cats absorbing fat. Thoracic duct cannulated before giving the fat meal.
Middle: Groups of cats absorbing fat. Thoracic duct cannulated after fat absorption had commenced.
Below: Group of post-absorptive cats.

The same regression was derived during fat absorption and is given by the equation:

T.D.P.P.=18·54+7·03 (P.P.P.—9·39). P.=0·01.

Fig. 6 shows these regressions graphically. The slopes of the two regression lines are significantly different, (P.=0·001) and during fat absorption the thoracic duct lymph phospholipide levels are seen to be higher than those of the plasma. This reversal in the concentration gradient from that seen in the post-absorptive state shows that much of the thoracic duct lymph phospholipide during fat absorption is being derived from a source other than the circulating plasma.

The changes in plasma-lymph cholesterol equilibria have also been plotted in Fig. 7. Three groups of cats have been included in this figure. The lower graph represents the post-absorptive relationships, which have previously been discussed. The middle graph shows the plasma-lymph total cholesterol equilibrium in a series of fat-fed cats whose thoracic ducts were cannulated after fat absorption had been proceeding for a considerable period. The upper graph is the regression of lymph total cholesterol on plasma total cholesterol in a series of fed cats which received a fat meal after the thoracic duct was cannulated. The regression equations, plotted in Fig. 6, are given:

Post absorptive. T.D.T.C.=46·21+0·328 (P.T.C.—99·32) (P=0·001).

Fat absorption. T.D.T.C.=93·27+0·490 (P.T.C.—110·13) (P.=0·001).

Fat absorption with thoracic duct cannulated. T.D.T.C.=110·78+0·653 (P.T.C.—95·22) (P.=0·02).

where T.D.T.C.=thoracic duct total cholesterol.

P.T.C.=plasma total cholesterol.

No significant difference exists between the slope of the three regression lines, but the mean level of cholesterol in the lymph of each absorptive group is significantly higher than that of the post-absorptive series. From the series

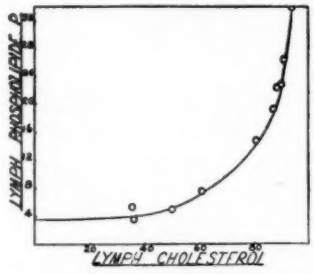


**Fig. 8.** The relationship of total cholesterol (mg. p.c.) to phospholipide phosphorus (mg. p.c.) in the thoracic duct lymph of the cat during fat absorption.

of results available the amount of total cholesterol added to the circulating plasma during fat absorption did not raise the plasma levels sufficiently to produce a significant difference between the groups.

The relationship of total cholesterol and phospholipide phosphorus in the thoracic duct lymph before and during fat absorption was next examined. From considerations of the concentration changes alone, the ratio of cholesterol to phospholipide phosphorus in the lymph was far from constant during fat absorption and the extent of the change is seen in Fig. 8. As the total fatty acid content of the lymph rose, the phospholipide fraction increased. The greatest increases in lymph phospholipide occurred at the highest levels of total fatty acid, whereas the rate of increase of cholesterol tended to be more uniform. At high levels of lymph total fatty acid, the cholesterol/phospholipide ratio decreased rapidly. As with the lymph phospholipide/total fatty acid ratio, there was no constancy in the lymph cholesterol/phospholipide ratio during fat absorption. The gross alteration that occurs at high fat levels is due apparently to the much more significant metabolic relationship existing between fat absorption and phospholipide formation. The same qualitative changes occurred in the thoracic duct lymph of both the rabbit and rat during the absorption of a fat meal.

*Thoracic duct phospholipide output during fat absorption.*

The highly significant correlation between the total fatty acid and phospholipide concentration of the thoracic duct lymph during fat absorption is reflected in changes in the hourly output of phospholipide. In Table 5 the output of phospholipide in the thoracic duct lymph of cats, rabbits and rats is given for

TABLE 5.

*The amount of phospholipide recoverable in the thoracic duct lymph during fat absorption.*

| Experiment No. and duration | Plasma volume ml. | Plasma phospholipide concentration mg. p.c. lecithin | Total circulating plasma lecithin mg. | Total thoracic duct lymph lecithin output mg./10 hr. | p.c. of circulating plasma lecithin recovered in lymph |
|---|---|---|---|---|---|
| CATS—10 hours | | | | | |
| 1 | 84·0 | 170·2 | 143·0 | 78·0 | 54 |
| 2 | 133·0 | 195·0 | 259·0 | 152·0 | 59 |
| 3 | 189·0 | 237·5 | 450·0 | 243·0 | 54 |
| RABBITS—8 hours | | | | | |
| 1 | 72·0 | 107·0 | 77·0 | 36·0 | 47 |
| 2 | 110·0 | 110·0 | 121·0 | 15·0 | 12 |
| 3 | 72·0 | 52·5 | 38·0 | 30·0 | 79 |
| RATS—10 hours | | | | | |
| 1 | 7·2 | 142·5 | 10·0 | 48·0 | 462 |
| 2 | 8·6 | 160·0 | 14·0 | 87·0 | 632 |
| 3 | 8·2 | 85·0 | 7·0 | 71·0 | 1016 |

periods of 8–10 hours following the administration of a fat meal. The total lymph lecithin output is compared with the total circulating plasma lecithin. In the anaesthetized cat, slightly more than half the amount of circulating plasma phospholipide is recoverable in the thoracic duct lymph in a 10-hour period during fat absorption. Slightly less was recovered in the rabbit during an 8-hour collection period. In the unanaesthetized rat, the amounts of phospholipide recovered in the lymph over a 10-hour period during fat absorption amounted to 5–10 times the total circulating plasma phospholipide. These figures do not represent all newly synthesized phospholipide, as a proportion of that present in the lymph originates from the plasma as part of the capillary filtrate. Very large increments of newly synthesized phospholipide, however, are being added to the plasma through the thoracic duct lymph while fat absorption is proceeding.

*Recovery of administered fat in the thoracic duct.*

In the unanaesthetized rat an attempt was made to quantitate the amount of fat recoverable in the thoracic duct lymph following the feeding of olive oil. The 10-hour period following the fat meal was taken, and the results are given in Table 6. In three experiments the percentage recovery was 45, 41 and 79. This figure by no means represented the complete amount of fat carried by the thoracic duct, as the lymph total fatty acid concentration at the end of 10 hours was still quite high, being respectively 21, 45 and 67 m.eq./litre in each subject and fat absorption was still proceeding. The percentage recoveries of fat in the anaesthetized cat and rabbits were strikingly lower (Table 6). The small recovery compared with the rat appears due chiefly to the very much lower rates of lymph flow in these anaesthetized animals, which, on a weight basis, represent only about

TABLE 6.

*The recovery of fat in the thoracic duct lymph following the administration of olive oil by stomach tube.*

| Experiment | Total given mg. | Total recovered mg. | p.c. recovered | Maximum lymph total fatty acid concentration m.eq./l. | Maximum lymph flow ml./kg./hr. |
|---|---|---|---|---|---|
| CATS—10 hours | | | | | |
| 1 | 13725 | 2905 | 21 | 222·0 | 1·2 |
| 2 | 8235 | 465 | 6 | 158·5 | 1·0 |
| 3 | 10431 | 654 | 6 | 71·6 | 4·7 |
| RABBITS—8 hours | | | | | |
| 1 | 8235 | 162 | 2 | 36·9 | 1·8 |
| 2 | 9150 | 46 | 0·5 | 20·0 | 0·8 |
| 3 | 9150 | 94 | 1 | 45·6 | 1·1 |
| RATS—10 hours | | | | | |
| 1 | 915 | 411 | 45 | 211·4 | 5·8 |
| 2 | 915 | 377 | 41 | 78·8 | 21·4 |
| 3 | 915 | 719 | 79 | 267·4 | 21·1 |

one-twentieth of the flow rates recorded in the rat. In the cat and rabbit, the effect of anaesthesia on digestion, absorption and rate of lymph flow was not assessable.

## DISCUSSION.

*Plasma-lymph lipide equilibria.* In the post-absorptive state, the level of lipides in the circulating plasma is always higher than that in the lymph, suggesting that when a steady state exists, the lymph lipides have origin in their plasma counterparts. As this concentration gradient closely resembles the plasma-lymph protein relationships in both the thoracic and cervical duct lymph, it seems likely these lymph lipides represent the lipo-protein association with the plasma globulins, and as such would take part in the extravascular space-lymphatic circulation. This significant plasma-lymph interdependence is disturbed once the lymph receives exogenous lipide additions as occurs during fat absorption.

Transfused lipides rapidly leave the circulating plasma when lymph of high fat content is given intravenously. Some of the fat which escapes from the plasma into the extravascular space is returned to the circulating blood by way of the lymphatics. The level of fat in the plasma is apparently regulated within fairly narrow limits and a hyperlipaemic state in the normal animal does not persist for any appreciable time. Marble, Field, Drinker and Smith (1934) studied the course of the plasma and lymph lipides in the dog following the transfusion of lipaemic lymph and fat emulsions. They found small but significant increases in the lipide content of the cervical duct lymph after transfusion. Similar plasma-cervical duct lymph equilibria were reported in their post-absorptive dogs to those recorded here in the cat. The plasma and thoracic duct lymph levels are seen to vary appreciably between the different species. Cats had significantly higher levels of cholesterol and phospholipide in both plasma and lymph than the rabbit and rat. The group of rabbits fed on a vegetable diet had particularly low levels of lipides in both the plasma and thoracic duct lymph, reflecting the low intake of dietary fat.

*The interrelationships of the plasma and lymph lipides.* Since the work of Mayer and Schaeffer (1914) and their concept of lipide constants, investigations have been directed towards the relationships of the lipide fractions. In particular, phospholipide and cholesterol have been considered important in influencing the physico-chemical properties of biological membranes and in the development of such pathological states as atherosclerosis. Peters and Man (1943) studied a large series of normal humans in the post-absorptive state and found a constant ratio between the serum cholesterol and phospholipide, but could show no definite correlation between free fat and either cholesterol or lipide phosphorus. In the post-absorptive cat, a significant relationship is seen to exist between the lipide fractions of the plasma and lymph, and the importance of the plasma as a source

of lymph lipides is indicated. The lipide molecules in the plasma of the post-absorptive animal appear to pass through the capillary membrane to about the same extent as the plasma globulins. The concentration of lipide in the lymph is thought to be dependent on the relative ease with which the different lipoproteins can pass through the capillary membrane and the proportion of their constituent lipides. In the cat and the rabbit, the phospholipide/cholesterol ratio was significantly greater than 1 in both the plasma and the lymph before and during the absorption of fat.

*Lymph lipides during fat absorption.* The large volume of work presented on fat absorption has established the qualitative significance of the role played by the thoracic duct. The results of Bloom, Chaikoff, Reinhardt, Entenman and Dauben (1950), using $C^{14}$ labelled palmitic acid, indicated that up to 92 p.c. of the labelled fatty acid could be recovered in the thoracic duct lymph in 19–24 hours following its administration. The percentage recoveries in the rats reported here agree substantially with these findings. The effect of anaesthesia on fat absorption is reflected in the low percentage recoveries found in the cat and are similar to the results of Eckstein (1925) and Little and Robinson (1941). In the anaesthetized rabbit a very small percentage recovery of the administered fat meal was obtained.

The significance of the lymph lipide relationships occurring during fat absorption indicated an active participation of phospholipide in this process. Bloom, Chaikoff, Reinhardt and Dauben (1951) showed that as much as 96 p.c. of $C^{14}$ labelled fatty acids were recovered in the lymph in forms other than phospholipide. In this series about 10 p.c. of the fat carried by the thoracic duct lymph was combined as phospholipide. These results confirm that the phospholipides of the thoracic duct lymph, in themselves, are not significantly involved with the transport of absorbed fat. The work of Sinclair (1929), Schmidt-Nielson (1946) and Reiser (1953) point to the participation of phospholipides as essential intermediaries in fat absorption. This concept was not supported by Zilversmit, Chaikoff and Entenman (1948), who were unable to show any increase in the rate of turnover of phospholipide in the intestine during fat absorption. The highly significant regression of lymph phospholipide on lymph total fatty acid infers an active metabolic association between the two lipide fractions. Bollman, Flock, Cain and Grindlay (1950) showed in the dog that an increase in thoracic duct phospholipide occurred during fat absorption and that this phospholipide did not originate in the hepatic lymph. The intestinal mucosa follows as the obvious site for the synthesis of this thoracic duct phospholipide. The function of this increased lymph phospholipide during fat absorption is not known. It seems likely that these hydrophilic lipides would play an important part in maintaining as a stable emulsion, the large amounts of neutral fat appearing in the lymph during fat absorption.

In no instance during absorption have plasma phospholipide levels been

found to exceed those in the thoracic duct lymph, when this is diverted from the circulating blood. This finding does not support the contention of Frazer (1949) that, during fat absorption, the plasma phospholipide levels are usually higher than those of the lymph. The relationship of the lymph phospholipide to that of the circulating plasma is of interest. Fishler, Entenman, Montgomery and Chaikoff (1943), Entenman, Chaikoff and Zilversmit (1946), and Goldman, Chaikoff, Reinhardt, Entenman and Dauben (1950), using $P^{32}$ and $C^{14}$ labelled palmitic acid, concluded that the liver was the only site of formation of plasma phospholipides in the fasted dog. Bollman, Flock, Cain and Grindlay (1950) suggested that during fat absorption the intestinal mucosa was a source of plasma phospholipides. The very large increments of phospholipide of extrahepatic origin that are delivered to the circulating plasma during fat absorption make it apparent that much of the circulating plasma phospholipides at any time in the active animal will have an extrahepatic origin, presumably in the intestinal mucosa. It is not known, however, whether the phospholipide synthetized in the intestinal mucosa is identical with that formed in the liver. It is likely that differences in protein association and fatty acid composition would profoundly influence the biological activity of the phospholipide molecule, and those of hepatic origin may participate to a greater extent in the extravascular space-lymphatic circulation.

The increase in cholesterol output by the thoracic duct during fat absorption was a consistent finding in the three species. Its significance in affecting the fatty acid content of the lymph was only slight when compared with the effect of phospholipide. Widal, Weill and Laudat (1912), Terroine (1914), Milbrandt (1930), and Brockett, Speirs and Himwich (1934) all found an increase in the concentration of blood cholesterol during fat absorption. Brockett *et al.* (1934), concluded that cholesterol aided in the absorption of fatty acids. Other workers —Bloor (1915), Bang (1918), Hiller, Linder, Lundsgaard and Van Slyke (1924) and Blix (1926)—reported an irregular or no increase in cholesterol occurring with fat absorption. Studying the lymph lipides of the dog during fat absorption, Bollman *et al.* (1950), noted that a small rise of cholesterol occurred, but doubted its significance. These workers do not seem to have taken into account the flow factor when assessing the significance of concentration changes. In the cat and rabbit an increase in the cholesterol content of the lymph was indicative of an increased lymph cholesterol output. In the rat, the lymph cholesterol concentration fell, but due to the striking increase in lymph flow associated with fat absorption, the cholesterol output was increased. The significance of the regression of lymph total cholesterol on total fatty acid illustrates the interrelationship existing between these two fractions during fat absorption. Cholesterol is absorbed from the gut in the presence of fat and bile salts and appears in the thoracic duct lymph. The amount absorbed is considered to depend on the quantity of fatty acid accompanying it as the cholesterol is more or less

completely esterified (Sano, 1922). The suggestion by Burger (1928) that the cholesterol increase that occurs with fat absorption might be the result of a re-absorption of cholesterol excreted by the bile and intestinal mucosa seems likely. The cholesterol increase occurred to a greater degree in the cat than in the rabbit and rat, and has probably a passive association with fat absorption, rather than an active metabolic one.

## SUMMARY.

The concentration of lipides in the plasma of post-absorptive cats was found to be higher than that in the thoracic duct lymph. The thoracic duct lymph contained higher levels of lipide than did the lymph of the cervical duct. The concentration gradient existing between the lipides of the plasma and lymph was similar to that of the plasma and lymph globulins, and it is considered some plasma lipides are engaged in an active extravascular space-lymphatic circulation as occurs with protein.

When homologous lymph of high fat content was given intravenously in cats, plasma lipide levels returned to normal in about 2 hours. There was a rise in the thoracic duct lymph lipide concentration which occurred about half an hour after the transfusion began.

The concentration of lipide in the thoracic duct lymph of the post-absorptive cat was significantly related to the concentration of lipide in the circulating plasma. The level of total fatty acid in both the thoracic duct lymph and plasma was highly correlated with the amount of phospholipide present, and to a lesser degree with the concentration of total cholesterol. This significant interrelationship was maintained during fat absorption. It is considered that an active metabolic relationship exists between fat absorption and phospholipide synthesis whereas the increased cholesterol output by the thoracic duct lymph is thought to be a passive association following the absorption of fat.

The output of lymph total fatty acid, phospholipide and cholesterol increased during fat absorption. In the unanaesthetized rat, the fat recovered in the thoracic duct lymph during a 10-hour period following the administration of 1 ml. of olive oil by stomach tube represented some 41 to 79 p.c. of the total amount of fat given. In the anaesthetized cat and rabbit the recoveries were much lower. Following a meal of olive oil 3 ml./kg., 6 to 21 p.c. of the administered fat was recovered in the cat during 10 hours, and ½ to 2 p.c. in the rabbit in 8 hours.

The amount of phospholipide collected in the thoracic duct lymph during the 10 hours following a meal of olive oil was estimated as up to 10 times the total amount of circulating plasma phospholipide in the rat and about half the circulating plasma phospholipide in the cat and rabbit. This phospholipide increase was thought to originate in the intestinal mucosa, and the significance of the intestine as a source of plasma phospholipide in the active animal is discussed.

In the rat, only about 10 p.c. of the fat present in the thoracic duct lymph was combined as phospholipide. Some results for plasma and lymph lipide concentrations in the cat, rabbit and rat, under different dietary states, are presented.

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# AN ANALYSIS OF SOME FACTORS IN THE CARDIOVASCULAR RESPONSE TO ANAEMIA IN THE UNANAESTHETIZED RABBIT

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Blood volume changes, doubtless, influence the cardiac output in the normal and abnormal animal; however, little work has been done in the intact animal to determine their physiological importance. In the present work, an extension of that previously done on the circulatory changes in unanaesthetized anaemic rabbits (Darian Smith and Simmonds, 1954), the effects of changes in blood volume and haemoglobin concentration on the cardiac output and right atrial pressure, and their interaction in the "chronically" anaemic animal were investigated. In addition, experiments to determine the circulatory response to sudden changes in haemoglobin and blood volume were carried out.

## METHODS.

All experiments were done on unanaesthetized rabbits, of mixed breed, varying in weight from 1·3 to 2·5 kg.

The animals were made anaemic by daily bleeding of 14 ml./kg. body weight until the required haemoglobin concentration was reached.

Cardiac output determinations were made by the direct Fick method: details of the techniques used are published elsewhere (Korner and Darian Smith, 1954). Blood volumes were determined by the T1824 dye method, as previously described, except that more dye was injected (2 ml. of aqueous T1824 containing 2,500 mg./litre), and the separate blood sample volumes were reduced to 0·6 ml., the dye being extracted, using Allen's method (1951), from 0·2 ml. plasma.

Haemoglobin, and oxygen capacity determinations in the anaemic animals, were made with the cyanhaemoglobin method (Drabkin and Austin, 1935), using 0·2 ml. blood, the optical density being read at 540 $m\mu$ on a Beckman quartz spectrophotometer.

Details of the statistical procedures used are fully described by Fisher (1950).

Mean values are expressed together with the standard error of the mean.

## RESULTS.

### *"Chronic" anaemias.*

In a group of normal animals, previously reported (Korner *et al.*, 1954) analysis of the two separate factors required to be measured in determining the

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cardiac output by the direct Fick method proved useful. The same analysis has been used in these experiments and comparison with the former has demonstrated the similarity of the circulatory response of the normal and severely anaemic animal to certain physiological variations, which were measured. In the "chronically" anaemic animals all determinations were made 24 to 48 hours after the last haemorrhage.

*Oxygen consumption.* In both the normal and the "chronically" anaemic animal it was shown that the oxygen consumption was directly related to the body weight, and inversely related to the room temperature over the range 15°–25° C. This latter relationship suggested that the temperature "comfort zone" of the rabbit was near the upper limit of this temperature range, which accords with the finding in man, in the Australian climate (Kellerman, 1954). In addition in anaemic animals, the effect of a third variable, haemoglobin concentration, was not significant. Thus the oxygen consumptions of normal

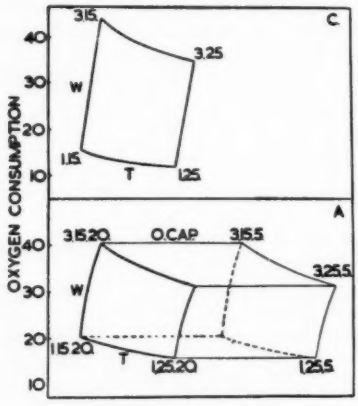


**Fig. 1.**

**Fig. 1.** Illustrates the similarity of the relations of weight (W.) in kg., and room temperature (T.) in degrees C., with the oxygen consumption (S.T.P.) in ml./min., in normal (upper) and anaemic rabbits (lower). In the latter the independence of oxygen consumption and oxygen capacity is also indicated. Co-ordinates of each point are in the order W., T., O.Cap.

and anaemic animals were essentially similar, being unaffected by changes in the haemoglobin concentration. These interrelations are illustrated in Fig. 1 (haemoglobin concentration being expressed as the oxygen capacity ml./100 ml. blood), and the multiple regression equations from which they are constructed given in Table 1.

TABLE 1.

*Regression equations relating oxygen consumption A-V oxygen difference, and cardiac output to the various factors discussed in the text, in normal and "chronically" anaemic rabbits.*

| | | No. of results | Regression equations | Significance of regression coefficients $b_1$ p= | $b_2$ p= | $b_3$ p= |
|---|---|---|---|---|---|---|
| Oxygen consumption | Normal | 55 | log O.C.=1·386+0·96 (log W. −0·270)−0·47 (log T.−1·302) | 0·001 | 0·001 | |
| | Anaemic | 50 | log O.C.=1·385+0·64 (log W.−0·252) −0·50 (log T.−1·331) +0·007 (log O.Cap.−1·000) | 0·001 | 0·01 | 0·8 |
| A-V oxygen difference | Normal | 54 | A.V.O.=6·1+0·15 (O.C.−25·2) +0·24 (O.Cap.−16·4) −0·92 (W.−1·9) | 0·001 | 0·01 | 0·2 |
| | Anaemic | 50 | A.V.O.=5·0+0·10 (O.C.−24·9) +0·27 (O.Cap.−10·9) −1·42 (W.−1·8) | 0·01 | 0·001 | 0·02 |
| Cardiac output | Normal | 55 | log C.O.=2·618+0·69 (log W.−0·276) −0·57 (log O.Cap.−1·213) −0·49 (log T.−1·302) | 0·001 | 0·07 | 0·02 |
| | Anaemic | 43 | log C.O.=2·734+0·60 (log W.−0·252) −0·71 (log O.Cap.−0·96) +0·52 (log B.V.−1·981) | 0·05 | 0·001 | 0·05 |
| | | 43 | *log C.O.=2·734+0·71 (log W.−0·252) −0·48 (log O.Cap.−0·96) | 0·001 | 0·001 | |

* See Figure 4.

O.C.=Oxygen consumption (ml./min.).
W.=Body weight (kg.).
T.=Room temperature (° C.).
A.V.O.=A-V oxygen difference (vols. p.c.).
O.Cap.=Oxygen capacity of blood (vols. p.c.).
C.O.=Cardiac output (ml./min.).
B.V.=Blood volume (ml.).

*Arterio-venous oxygen difference.* Even within the range of haemoglobin concentrations occurring in normal animals it was found that the A-V oxygen difference was significantly related to the oxygen capacity, decreasing as the oxygen capacity fell. This was, in addition, readily demonstrated in the anaemic animals. The rates of change of A-V oxygen difference with oxygen capacity were similar in both cases, demonstrated by the corresponding regression co efficients in the two regression equations (Table 1).

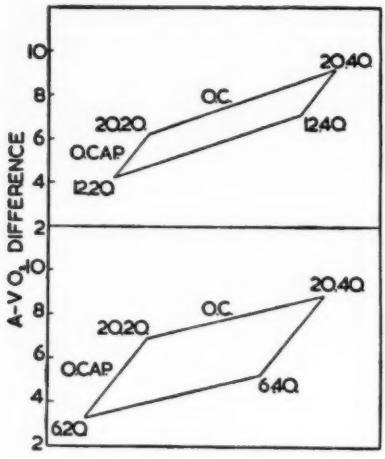


Fig. 2.

**Fig. 2.** Illustrates the similarity of the relations of oxygen capacity (O.Cap.) in ml./100 ml. of blood, and oxygen consumption (O.C.) in ml./min., with the A-V oxygen difference in ml./100 ml. blood in normal (upper) and anaemic animals (lower). The co-ordinates are in the order O.Cap., O.C. Mean body weights of the two series are considered (see regression equations).

A less obvious relationship, demonstrated in the normal series of animals, was the interdependence of A-V oxygen difference and the animal's oxygen consumption. This was interpreted to mean that physiological fluctuations in the animal's metabolism did not affect the cardiac output, but rather varied the percentage oxygen utilization of the blood. This same important mechanism was found to function in the anaemic animal, a rather surprising finding in view of the already high percentage oxygen utilization in these animals. These interrelations are illustrated in Fig. 2, and the corresponding equations are seen in Table 1.

*Cardiac output.* The rise in the cardiac output in haemorrhagic anaemias in rabbits has been previously reported (Darian Smith, *et al.*, 1954), the results being in general agreement with findings in man (Sharpey-Schafer, 1944; Bran-

non, Merrill, Warren and Stead, 1945). However, there, the relations between cardiac output, body weight, and haemoglobin concentration only were considered.

In the present experiments the separate effect of an additional variable, the blood volume, has been investigated. Fig. 3 illustrates the separate effects of

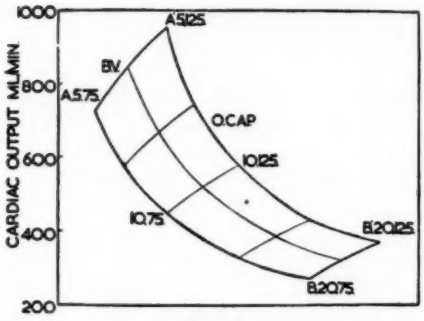


Fig. 3.

**Fig. 3.** Demonstrates the separate effects of oxygen capacity (O.Cap.) in ml./100 ml. blood, and blood volume (B.V.) in ml., on the cardiac output in anaemic animals. Co-ordinates are given in the order O.Cap., B.V. The animal considered has the mean weight of the series (1·8 kg.).

changes in oxygen capacity and blood volume on the cardiac output. The lines AB, A′B′ indicate the effect of change in oxygen capacity with a constant blood volume of the value indicated by the co-ordinates. The shorter lines, AA′, BB′, demonstrate that, at any particular oxygen capacity, an increase in blood volume within the physiological range was associated with an increase in cardiac output, the effect being greater in the more anaemic animals.

The change in blood volume in haemorrhagic anaemias in rabbits has been reported elsewhere. There was a progressive reduction in blood volume with an increasing degree of anaemia, the plasma volume remaining constant except for a slight expansion in the most severe anaemias, and the total blood volume approximately reflecting the change in red cell volume.

Fig. 4 illustrates the effect of the actual blood volume changes in these anaemic animals on the cardiac output. The heavy line is the simple logarithmic relation of cardiac output to haemoglobin concentration, expressed as oxygen capacity. This has been superimposed on the family of curves ABB′A′, from Fig. 3, constructed for constant blood volumes. If the blood volume were to remain constant, the expected rise in cardiac output with a falling haemoglobin concentration would be along the line B′A′. The depressant effect of the blood volume change on the cardiac output is thus readily seen.

In the above discussion and graphs the separate effect of a change in weight has not been considered, but, in order to simplify presentation, has been taken

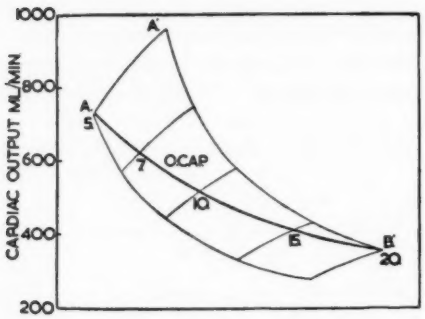


Fig. 4.

**Fig. 4.** Demonstrates the actual cardiac output at any level of oxygen capacity—heavy line. This has been superimposed on the family of curves illustrated in Fig. 3, A'B'BA, to demonstrate the depressant effect of the change in blood volume. If the blood volume were to remain constant and the oxygen capacity were 5 ml./100 ml. blood, the expected cardiac output would be at A' rather than the lower value near A. Ordinates give value of oxygen capacity.

to be the mean of the series, 1·8 kg. The effect, however, is indicated in the regression equation in Table 1.

Korner *et al.* (1954), have shown a significant relationship in normal rabbits between cardiac output and room temperature; owing to the small range of the latter in the present experiments this relationship was not investigated.

*Right atrial pressure.* Elevation of the mean right atrial pressure in severe anaemias has been reported in man (Sharpey-Schafer, 1944) and in the rabbit (Darian Smith, *et al.*, 1954). The elevation of the cardiac output and mean right atrial pressure were significantly related. The variation in mean right atrial pressure at a particular haemoglobin concentration, due in part to the changes in blood volume, was not, however, significantly related to the cardiac output. This was probably due to the limited number of observations made.

*The time factor and the circulatory response to a sudden change in haemoglobin concentration.*

The time taken for the various circulatory responses, particularly the rise in cardiac output, when the haemoglobin concentration was suddenly changed was then investigated because it was thought that the interrelations of cardiac output, haemoglobin concentration, right atrial pressure and blood volume might be more readily investigated in acute experiments.

Six animals were used for the initial experiments. In three, made severely anaemic before the experiment, the cardiac outputs were determined immediately before and ½ hour after a transfusion of packed, cross-matched, red cells suspended in Ringer-Locke solution.

In the three remaining animals the haemoglobin level was reduced to about 8 gm./100 ml. before the experiment. A control cardiac output determination was done and the animal then bled 5–6 ml./kg. every 20 minutes over a period of 80–100 minutes. This disturbed the animals very little. They were not shocked by the procedure, as was indicated by their alertness and the fact that their mean systemic blood pressure never fell more than a few mm. Hg. This procedure was possible because of the extremely rapid rate of replacement of lost plasma in the rabbit. A second cardiac output determination was done one hour after the last haemorrhage, when the plasma volume had been largely replaced. This was checked by a blood volume determination. Thus, in both these groups of experiments, a rapid, marked change in haemoglobin concentration was produced with a minimal change in plasma volume.

The results were clear-cut. At the lower haemoglobin level, both the cardiac output and right atrial pressure were higher significantly than in the less anaemic animals. The method of producing the change in haemoglobin concentration did not affect the results significantly, as demonstrated by simple analysis of variance. The mean change in oxygen capacity was from 9·8 ml. to 6·1 ml./100 ml. blood. The mean cardiac output rose from 469±128 ml./minute to 837±161 ml./minute and the mean right atrial pressure from 6·5 cm. $H_2O$ to 9·0 cm. $H_2O$.

The above experiments indicated that the change in cardiac output, with a change in haemoglobin concentration, occurred within one hour. To determine if it were more rapid, it was necessary to take into account the associated blood volume change inevitable immediately after a haemorrhage. The experiments were so designed that cardiac outputs and blood volumes were measured within 10 minutes after completing a moderate haemorrhage. Thirteen animals were used. Before beginning the experiment, the haemoglobin concentration was reduced "chronically" to the required level, the group being constructed so that the scatter in the range of anaemias was even. A control cardiac output was done, then 8–9 ml. of blood/kg. body weight were removed rapidly through the carotid catheter and the volume measured to the nearest 0·1 ml. Within ten minutes of completing the haemorrhage the cardiac output was again determined. A further 6–8 ml./kg. were then removed and the cardiac output again determined within ten minutes of completing the second haemorrhage. Fifteen to twenty minutes later a plasma volume determination was made. Having an accurate measure of the final red-cell volume, the volume of red cells removed with each haemorrhage, and the haematocrit value at the time of each cardiac output determination, the blood volume at that instant was calculated.

Analysis of the results showed that the cardiac output in these acute experiments was affected by changes in both blood volume and haemoglobin concentration in a manner not differing significantly from that found in the "chronically" anaemic animals. This similarity is evident on comparing the two regression equations of cardiac output on these factors in Table 2. The inference from

TABLE 2.

*Regression equations, relating oxygen consumption, cardiac output, and mean right atrial pressure to the factors discussed in the text, in the "acute" experiments.*

| | No. of Results | Regression equations | Significance of regression coefficients $b_1$ p= | $b_2$ p= | $b_3$ p= | $b_4$ p= |
|---|---|---|---|---|---|---|
| Oxygen consumption | 38 | log O.C.=1·543+0·42 (log W.−0·228) +0·29 (log O.Cap.−0·996) | 0·01 | 0·001 | | |
| Cardiac output | 38 | log C.O.=2·664+0·35 (log W.−0·228) −0·82 (log O.Cap.−0·996) +1·06 (log B.V.−1·912) −0·228 (log R.A.P.−0·763) | 0·3 | 0·001 | 0·01 | 0·1 |
| | 38 | log C.O.=2·664+0·34 (log W.−0·228) −0·67 (log O.Cap.−0·996) +0·68 (log B.V.−1·912) | 0·3 | 0·001 | 0·05 | |
| Mean right atrial pressure | 38 | log R.A.P.=0·763+0·04 (log W.−0·228) −0·66 (log O.Cap.−0·996) +1·64 (log B.V.−1·912) | 0·6 | 0·001 | 0·001 | |

O.C.=Oxygen consumption (ml./min.).
W.=Body weight (kg.).
C.O.=Cardiac output (ml./min.).
O.Cap.=Oxygen capacity of blood (vols. p.c.).
B.V.=Blood volume (ml.).
R.A.P.=Mean right atrial pressure, cm. $H_2O$.

these results is that the change in cardiac output occurring in response to a rapid fall in haemoglobin concentration is independent of time, at least after 5–10 minutes.

The simple relationship between cardiac output and mean right atrial pressure, plotted logarithmically, was significant in these acute anaemias. When the simultaneous effects of changes in haemoglobin concentration, blood volume and right atrial pressure on cardiac output were taken into account by multiple regression, it was found that the relationship between cardiac output and right atrial pressure was no longer significant. That is, if the other factors remained constant, changes in right atrial pressure over the range measured did not significantly affect the cardiac output. Again, as in the "chronic" anaemias, this was probably due to the limited number of observations.

A highly significant correlation was demonstrated between right atrial pressure and both oxygen capacity and blood volume, as might be expected from the immediately preceding results. These relationships are illustrated in Fig. 5.

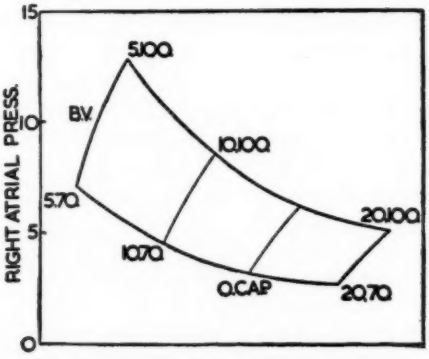


**Fig. 5.** Illustrates the relation of oxygen capacity (O.Cap.) in ml./100 ml. blood, and blood volume (B.V.) in ml., to the mean right atrial pressure in cm. $H_2O$. Co-ordinates are in the order O.Cap., B.V. Weight =1·7 kg.

The other important change occurring in these experiments was in relation to the oxygen consumption of the animals. In the normal and mildly anaemic animals marked reduction in blood volume did not significantly affect the animals' oxygen consumption. In the severely anaemic animals, however, there was a considerable temporary drop in oxygen consumption, but when the plasma volume had returned to normal the oxygen consumption had returned to its previous level. The interpretation of this relationship was not evident.

## DISCUSSION.

The purpose of analysing the interrelations of the various cardio-vascular and respiratory factors in the "chronic" group of anaemic animals was to compare them with similar relations in normal animals. The outstanding feature of such analyses was the demonstration of the essential parallelism in various cardio-vascular changes in the two groups. The temperature regulating mechanism in the anaemic animals, measured by the significant relation between the animals' metabolic rate and room temperature, was normal. Similarly, the ability of the normal animal to cope with variations in oxygen requirements by correspondingly varying the percentage oxygen utilization was maintained in the anaemic animal in spite of the already high percentage oxygen utilization.

The significance of these findings is, perhaps, made more clear by comparing them with the corresponding relations in the acutely anoxic rabbits (Korner, 1954). Acute anoxia, in the rabbit, apparently damages the heart muscle sufficiently to cause cardiac failure, with a significant, large, fall in cardiac output. Under these circumstances the relations between room temperature and oxygen consumption, and A-V oxygen difference and oxygen consumption, were lost. Similar findings were obtained by Hemingway and Nahas (1952).

Analysis of the importance of time in the circulatory response to a rapid change in haemoglobin concentration was of interest in relation to a suggestion made by McMichael (1947) that an endocrine factor was possibly responsible for the 24-hour delay in the rise in cardiac output in patients following a haematemesis. Although the present work still offers no evidence about the rate of response during the first 10 minutes after a haemorrhage, our results suggest that it is very rapid. In addition, a purely mechanical explanation for the delayed rise of the cardiac output in humans might be made on the basis of our animal findings. In the rabbit plasma replacement after haemorrhage is practically complete within an hour, whereas in the human fluid replacement may still be incomplete after 24 hours. Thus, the slow rise in cardiac output after haemorrhage might well be due to the effect of the low blood volume, rather than a true, delayed response to the fall in haemoglobin concentration. At least in the rabbit the rapidity of the circulatory response to change in haemoglobin concentration suggested that it is mediated *via* a nervous mechanism rather than an endocrine factor.

In these experiments changes in haemoglobin concentration and blood volume accounted for a large part of the variation in two factors, namely, the cardiac output and the mean right atrial pressure. The cardiac output and mean atrial pressure were further demonstrated to be significantly related. It seems probable that changes in mean right atrial pressure were the immediate determinant of the cardiac output in these animals, and that the other factors influenced the cardiac output through their effect on the filling pressure of the heart.

Previously we had suggested that to maintain the high venous return in severely anaemic animals, when the blood volume was less than normal, the volume of the vascular circuit would need to be reduced, presumably by regional vasoconstriction. Further evidence that this is likely is the relationship between mean right atrial pressure and total blood volume. For a given increase in blood volume in the severely anaemic rabbits the rise in atrial pressure was approximately double that in normal rabbits. This was not due to cardiac incompetence, because the cardiac output increased correspondingly, but possibly was the result of a reduced venous volume.

The reason for the fall in the oxygen consumption in severely anaemic animals when the blood volume was suddenly reduced, is not clear. Possible explanations are that there was some temporary interference with oxygen transport to the tissues, or alternatively, a compensatory reduction in the tissue oxygen requirement, but the analyses of the available experiments offered little help in determining which factor was important.

## SUMMARY.

In unanaesthetized anaemic rabbits cardiovascular responses to certain physiological changes were normal. Temperature regulation and the cardiovascular responses to physiological variations in oxygen consumption and blood volume were similar to those in the normal animal.

The cardiovascular response to sudden change in haemoglobin concentration occurred within 10 minutes, any apparent delayed response following haemorrhage being due to the depressant effect of the associated reduced blood volume.

In acute and "chronic" anaemias analysis of factors affecting cardiac output demonstrated its relation to oxygen capacity, blood volume and the mean right atrial pressure. The latter was closely correlated with oxygen capacity and blood volume, both of which influenced cardiac output. The significance of these relationships has been discussed. The direct relation of the cardiac output to blood volume further demonstrated the competence of the heart in the anaemic animal.

*Acknowledgments.* My thanks are due to Dr. F. C. Courtice, in whose laboratory this work was done, for his interest and encouragement. I also wish to thank Dr. P. I. Korner for help with the design and analysis of experiments and much criticism, and Misses M. Woodward and P. Blakey for technical assistance.

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# STUDIES ON INTERMEDIARY IRON METABOLISM

## V. THE MEASUREMENT OF NON-HAEMOGLOBIN TISSUE IRON

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*(Accepted for publication 17th May, 1954.)*

The tissues of the liver, spleen and bone marrow are capable of storing iron in the form of specific organic compounds and may jointly contain as much as 25 p.c. of the total body iron under normal conditions. The amount of storage iron varies with sex and age (Bruckman and Zondek, 1939) and is also affected by diseases which change the normal pattern of iron metabolism (Finch *et al.*, 1950). Besides the storage iron compounds—ferritin and haemosiderin—the tissues of the storage organs also contain iron compounds common throughout the body, namely, haematin enzymes and haemoglobin. The concentration of haematin enzymes is fairly constant for a given kind of tissue, and they contribute only a negligible quantity of iron to the total. The concentration of haemoglobin, however, is subject to considerable variation even within the same organ. Under certain conditions the ratio of haemoglobin iron to storage iron may be quite high. It can be concluded that any analytical procedure for the estimation of storage iron should eliminate haemoglobin iron from the measurement.

Haemoglobin can be eliminated from the viscera by saline viviperfusion performed immediately before killing the animal (Whipple, 1926). This method is somewhat laborious and is not always entirely successful. Alternative methods of eliminating haemoglobin are based on its high resistance to acids and the contrasting low resistance of the storage iron compounds to same. The best known of these methods is that of Bruckman and Zondek (1939), who hydrolyzed tissue homogenates with trichloracetic acid after treatment with sodium pyrophosphate. More recently, Anderson (1950) described the extraction of non-haemoglobin tissue iron with 0·66 p.c. hydrochloric acid, carried out at 37° C. for 48 hours in a shaking machine. As this procedure requires special apparatus and is slow, an attempt was made to simplify the method. This paper reports the modification in detail and experiments for testing its validity.

[1]This work was undertaken during tenure of a National Health and Medical Research Council Fellowship.

## Materials and Methods.

*Handling of tissues.* Rat livers and spleens were mostly used in this work but some of the experiments were duplicated on mouse and rabbit livers.

After exsanguinating the anaesthetized animal the required organs are dissected and their surface dried with filter paper. They are stored in a freezing chamber and aliquots for analysis are cut only after the tissues are frozen. Aliquots between 0·1 and 0·3 gm. are weighed in small porcelain mortars and are refrozen by placing the mortars in the freezing chamber.

*Analysis of non-haemoglobin iron.*

The frozen aliquot is ground with a pestle. When the tissue becomes pasty, distilled water is added drop-wise from a Pasteur pipette until an even suspension is obtained. This requires about 5 ml. of distilled water. The suspension is now transferred with another Pasteur pipette into a 50 ml. volumetric flask. The mortar and the pestle are rinsed with approximately 20 ml. distilled water in four or five steps, each subsequent washing being united with the initial 5 ml. in the volumetric flask.

20 ml. 10 p.c. hydrochloric acid are added to the suspension and the contents of the flask made up to the mark with distilled water.

The flask is placed in a 85° C. waterbath for 30 minutes and is repeatedly inverted at 10-minute intervals.

The contents of the flask are transferred to a 50 ml. centrifuge tube which is spun for 15 minutes at 1,500 r.p.m.

A 10 ml. aliquot of the supernatant fluid and 2 ml. 40 p.c. trichloracetic acid are pipetted into a centrifuge tube. The tube is placed in the 85° C. waterbath for 15 minutes.

The tube is spun for 15 minutes at 3,000 r.p.m.

Duplicate aliquots of 5 ml. of the supernatant fluid are taken for colorimetry with orthophenanthroline.

Details of the colorimetric procedure are identical with that described in a previous paper (Kaldor, 1953). A blank solution is prepared with each batch of tissues to be analysed.

*Analysis of tissue aliquots and residues for total iron content.* In a number of experiments tissue aliquots and residues (obtained after centrifuging the hydrolysate — see "Analysis of non-haemoglobin iron") were wet ashed with 1·0 ml. sulphuric acid and 1·0 ml. perchloric acid. The clear digests were diluted with distilled water and the iron content measured colorimetrically.

## EXPERIMENTAL RESULTS.

In the present method the tissue homogenates are hydrolysed with more concentrated hydrochloric acid and at a higher temperature, but for a shorter period than in Anderson's method, i.e., 4 p.c. hydrochloric acid for 30 minutes at 85° C. instead of 0·66 p.c. hydrochloric acid for 48 hours at 37° C. This modification of Anderson's method was adopted after studying the resistance of haemoglobin to hydrolysis under varying conditions. Aliquots of haemolysed blood were hydrolysed with 1 p.c., 4 p.c. and 10 p.c. hydrochloric acid for 15, 30 and 60 minutes. The percentage of liberated iron was measured in each of these hydrolysates and the results are shown in Table 1.

TABLE 1.

*Percentage of total haemoglobin iron found in hydrolysates of haemolysed rat blood.*

| Concentration of HCl | Duration of hydrolysis 15 minutes | 30 minutes | 60 minutes |
|---|---|---|---|
| 1 p.c. | 6·0 | 6·8 | 7·8 |
| 4 p.c. | 6·3 | 6·4 | 7·9 |
| 10 p.c. | 15·2 | 16·9 | 19·5 |

It will be seen that while 1 p.c. hydrochloric acid liberates a small amount of iron, the amount of iron liberated is unchanged when the concentration of the acid is raised to 4 p.c. However, when the concentration is raised to 10 p.c. the amount of iron liberated is increased almost three-fold. It was on the basis of these findings that hydrolysis with 4 p.c. hydrochloric acid for 30 minutes was chosen.

When tissues are subjected to this treatment most of the tissue proteins are precipitated and come down in the residue when the hydrolysate is centrifuged. Some of the tissue proteins, however, remain in solution and, as their presence would interfere with the subsequent colorimetry, treatment with trichloracetic acid completes precipitation of the proteins. The iron content of this second precipitate is negligible and can be ignored.

The residue resulting from the first precipitation and centrifugation of the hydrolysate is rich in iron, the amount depending on the haemoglobin content of the tissue.

The supernatant of the hydrolysate contains all the hydrolysable iron, i.e., the ferritin and haemosiderin of the tissue.

TABLE 2.

*Total iron content and its subdivisions in liver specimens.*

| Specimen No. | No. of aliquots | Mean iron content (μg. Fe per mg. tissue) of hydrolysates | of residues | of digests | total | Ratio of residual to total |
|---|---|---|---|---|---|---|
| 1 | 4 | 107 | 27 | — | 134 | ·20 |
| | 2 | — | — | 138 | 138 | |
| 2 | 4 | 176 | 46 | — | 222 | ·21 |
| | 2 | — | — | 205 | 205 | |
| 3 | 4 | 308 | 19 | — | 327 | ·06 |
| | 4 | — | — | 336 | 336 | |
| 4 | 8 | 169 | 22 | — | 191 | ·12 |
| 5 | 6 | 177 | 55 | — | 232 | ·24 |
| 6 | 8 | 163 | 43 | — | 206 | ·21 |

Table 2 shows the results of iron estimations on six liver specimens which were taken from exsanguinated animals. Iron was measured in the hydrolysate and in the residues on aliquots from each specimen, and control measurements, to estimate the total iron, were made on wet ashed aliquots from three of the six specimens. The total iron content of fractionated aliquots (hydrolysable iron+residual iron) agrees well with the total iron content of wet ashed control aliquots. This indicates that all iron in the tissue is accounted for in the described procedure.

The wide variation in the amount of iron in the residue is probably due to individual variation in the degree of removal of the intra-hepatic blood during exsanguination. This interpretation was checked by adding haemolysed blood to a series of tissue aliquots before hydrolysis and comparing the iron content of the residues after hydrolysis with the iron content of the residues of hydrolysed control aliquots with no added blood. The difference between the readings represented the iron content of the added blood.

Statistical analysis of the results showed that the coefficients of variation ($\frac{\text{standard deviation}}{\text{mean}} \times 100$) of replicate measurements of non-haemoglobin iron ranged from 1 to 11 p.c. with a mean value of 6·8 p.c. No statistically significant difference was found in the iron content of aliquots of different weight or of aliquots taken from different parts of the same liver.

## DISCUSSION.

The method described here for the estimation of non-haemoglobin iron in tissues accepts the impossibility of completely exsanguinating tissue, and relies, as do other methods, on the differential effect of hydrolysis on haemoglobin and on tissue iron respectively. While the iron of haemoglobin is resistant to liberation by hydrolysis, the resistance is not absolute, and a small amount of haemoglobin iron is liberated. In the described method this has been estimated at between 6 and 7 p.c., and this amount represents an error of 0·5 to 2 p.c. in the total amount of hydrolysable iron in liver. This error is small compared with the total error of the measurements (mean coefficient of variation: 6·8 p.c.). In comparison with Anderson's method the modified procedure seems more economical. Aliquots of 0·1 to 0·3 gm. weight required for analysis leave the greater part of the tissue available for other chemical and histological tests, and, if necessary, could be repeatedly obtained from the same animal by laparotomy at various stages of an extended experiment. The modifications made in the technique of hydrolysis have shortened the procedure without affecting the accuracy of the method.

## SUMMARY.

A method is described for measuring non-haemoglobin iron in tissues. Iron is extracted from a tissue homogenate with 4 p.c. hydrochloric acid. The extract is made protein-free by precipitation with trichloracetic acid and its iron content is determined colorimetrically with orthophenanthroline. The overall error of the method calculated from replicate measurements, and expressed as the coefficient of variation, is 6·8 p.c.

*Acknowledgment.* The author wishes to acknowledge the technical assistance of Miss Marjorie Powell.

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# STUDIES ON INTERMEDIARY IRON METABOLISM

## VI. THE ABSORPTION AND STORAGE OF IRON IN EXPERIMENTAL ANAEMIA

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*(Accepted for publication 17th May, 1954.)*

McCance and Widdowson (1937) demonstrated that insignificant amounts of iron are excreted by the body under normal conditions and that excretion is only slightly increased when intravital haemolysis is induced experimentally. As a consequence of this relative inability of the body to excrete iron, iron absorption must be delicately regulated to ensure a physiologically adequate supply without permitting excessive accumulation in the tissues.

The nature of the regulating mechanism has been studied by a number of investigators. The work of Whipple's school (1942) demonstrated that whereas normal dogs absorb little dietary or orally administered iron, absorption increases 5 to 15-fold one week after the onset of anaemia induced by bleeding. They concluded that iron absorption is adjusted to need and is regulated by the size of the iron stores. However, Kinney, Hegsted and Finch (1949) found increased iron absorption in normal rats on a corn grit diet and showed that this was due to the low phosphorus content of the diet.

Radioactive iron isotope studies of Dubach, Callender and Moore (1948) demonstrated that patients with fever, untreated pernicious anaemia and refractory anaemia absorb more iron than they use for haemoglobin, and that patients with haemolytic anaemia may also absorb more iron than they require. They concluded that in these diseases patients may absorb considerable quantities of iron even though the iron stores are normal or increased in size.

These findings have recently been corroborated (Goldish and Aufderheide, 1953; Wallerstein and Robbins, 1953).

The purpose of this paper is to report the results of experiments which were performed on rats rendered chronically anaemic by repeated injections of phenylhydrazine. The results demonstrate increased absorption and storage of dietary iron in animals subjected to red-cell destruction and whose iron stores were not depleted.

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[1]This work was undertaken during tenure of a National Health and Medical Research Council Fellowship.

## Materials and Methods.

*Principle of the experiment.*

Phenylhydrazine produces anaemia due to increased red-cell destruction. Although the red cells destroyed by a single dose of the drug are rapidly replaced, anaemia can be prolonged by repeated controlled dosage. The animals invariably recover and show no after effects when treatment is discontinued. During the period of increased red-cell destruction the iron liberated from haemoglobin is diverted to the tissues, but during the recovery phase the same amount of iron must be reutilised for haemoglobin synthesis. Any difference between the iron content of the tissues of the recovered animals and that of the untreated controls must therefore be due to differences in intestinal iron absorption.

*General Plan.*

Seventy-two female rats, 9 to 12 months old and of 200 gm. ±20 gm. mean body weight, were divided into four equal groups as follows:—

*Group A.* Rats in this group served as controls, were untreated and received the usual rat diet.

*Group B.* These were treated with phenylhydrazine for 16 weeks (0 to 16th week of the experimental period).

*Group C.* This group was not treated with phenylhydrazine, but for a period of 4 weeks (12th to 16th week) the rat diet was supplemented by the addition of iron to the drinking water in the form of 2 p.c. ferrous ammonium sulphate.

*Group D.* These rats were treated with phenylhydrazine during the same period as group B (0 to 16th week) and their diet was supplemented with iron in the same manner as that of group C between the 12th and 16th week of the experimental period.

All animals were sacrificed between the 26th and 28th week from the beginning of the experiment, i.e., the rats rendered anaemic with phenylhydrazine for 16 weeks were allowed a period of 10 to 12 weeks for recovery.

*Control of haemoglobin value.*

Phenylhydrazine was given by intraperitoneal injections of a freshly prepared 2 p.c. solution of phenylhydrazine hydrochloride (solutions of phenylhydrazine oxidize rapidly and change into toxic compounds on standing). Injections were made at 2 to 5-day intervals and the amount injected varied between 0·2 and 0·5 ml. per rat. The dosage was designed to maintain the haemoglobin value of the rats at approximately 60 p.c. of the value of the controls. During the initial stages of treatment haemoglobin estimations were made on blood samples from each rat every 2 or 3 days. Later it was found that sufficient control was obtained by comparing the mean haemoglobin value of 4–6 rats from each group, selected at random.

To ascertain the return of the haemoglobin value during the recovery period to the level of that of the controls, rats from all four groups were tested three and eight weeks after the last injection. Recovery was complete after three weeks and the values were unchanged at eight weeks.

*Collection of samples and analytical methods.*

The rats were anaesthetized with ether. The chest was opened and blood was withdrawn from the heart with syringe and needle. The liver and the spleen were dissected and weighed. The blood samples were centrifuged and the sera of three rats were pooled in equal proportions. Latent iron-binding capacity was measured on aliquots of the fresh pooled sera. The remaining portion of the sera, the livers and the spleens were then frozen and serum iron and non-haemoglobin tissue iron analyses were made later. The methods of analysis were the same as previously described. (Serum iron and iron-binding capacity: Kaldor, 1953; Non-haemoglobin tissue iron: Kaldor, 1954.)

## RESULTS.

The rats treated with phenylhydrazine maintained their body weight throughout the experiment, and they did not differ in appearance or activity from the control rats. On dissection, however, a marked difference was observed between the size of the spleens of phenylhydrazine-treated rats and of the control rats. The mean body weights and the mean weights of the livers and spleens are shown in Table 1.

TABLE 1.

*Mean body weight of rats and the mean weights of the livers and spleens.*
(The figures shown are the mean±its standard error and the standard deviation.)

| Group | Body weight, gm. | Liver weight, gm. | Spleen weight, gm. |
|---|---|---|---|
| A | 207±5<br>20 | 6·00±·21<br>·91 | 0·65±·025<br>·11 |
| B | 204±3<br>12 | 5·88±·12<br>·51 | 0·90±·027<br>·11 |
| C | 197±3<br>12 | 5·63±·18<br>·78 | 0·62±·028<br>·12 |
| D | 208±4<br>16 | 6·42±·22<br>·92 | 1·44±·071<br>·30 |

The observed differences in the mean body weights and in the mean weights of the livers of the four groups cannot be correlated with the experimental procedures. The weight of the spleen, on the other hand, was significantly increased in all rats treated with phenylhydrazine (groups B and D) and this effect was enhanced by the administration of supplementary dietary iron (group D) even though supplementary dietary iron alone (group C) caused no change in the weights of the spleen.

In Table 2 are given the results of iron analyses on the sera, livers and spleens.

TABLE 2.

*The results of iron analyses on sera, livers and spleens.*
(The figures shown are the mean±its standard error and the standard deviation.)

| Groups | Serum iron μg. Fe per 100 ml. serum | Liver iron mg. | Spleen iron mg. | Liver iron+ spleen iron mg. |
|---|---|---|---|---|
| A | 260±9<br>32 | 3·05±·26<br>1·11 | 1·30±·13<br>·54 | 4·35±·36<br>1·54 |
| B | 343±15<br>37 | 5·30±·17<br>·74 | 4·75±·22<br>·92 | 10·05±·30<br>1·28 |
| C | 230±10<br>25 | 3·87±·22<br>·93 | 1·35±·14<br>·60 | 5·22±·30<br>1·28 |
| D | 382±14<br>33 | 6·61±·27<br>1·14 | 5·79±·27<br>1·15 | 12·40±·39<br>1·67 |

The following major changes are seen:

Both serum iron and tissue iron were considerably increased in all rats treated with phenylhydrazine (compare groups B and D with groups A and C, respectively).

The sum of the liver and spleen tissue iron (shown in the last column of the Table) was increased in those rats which received extra dietary iron (compare group C with group A and group D with group B). This iron fraction represents a very high percentage of the total non-haemoglobin iron of the body. The increase was more pronounced in the rats which received extra dietary iron simultaneously with phenylhydrazine (group D).

The mean total iron binding capacity of sera was aproximately 500 $\mu$g. in each group, i.e., unaffected either by treatment with phenylhydrazine or by iron feeding.

## DISCUSSION.

The results reported may be considered from two points of view:

1. An increase in iron absorption occurs in rats during phenylhydrazine-induced anaemia—a finding similar to that of Stewart, Vassar and Stone (1953).

2. An increase in iron absorption also occurs both in normal rats and in rats treated with phenylhydrazine when iron is added to the diet. In the latter group the increase due to dietary iron is additional to the increase observed after phenylhydrazine treatment.

The increased iron absorption in rats with phenylhydrazine anaemia is certainly not due to depleted iron stores because a large amount of haemoglobin iron was diverted to the stores during the anaemic phase. It is almost certain that the reduced haemoglobin value was the factor determining the rate of absorption, but the mechanism of its operation is difficult to explain. Granick (1949) has suggested that anoxia, due to anaemia, may alter the oxidation-reduction level of the mucosal cells of the gastro-intestinal tract. He believes that the passage of iron through the intestinal mucosa is generally governed by the rate at which ferrous iron can be oxidized to ferric iron of ferritin, and subsequently reduced again to the ferrous state before passing into the blood stream. Any alteration in the supply of oxygen to the cells could disturb this mechanism and so alter the rate of iron absorption.

The increase due to addition of iron to the diet cannot be related in any way to iron stores, or haemoglobin values. The factors which normally control the amount of iron absorbed (Granick, 1949; Drabkin, 1951) obviously must be dependent to some extent on dietary factors (Kinney, Hegsted and Finch, 1949) and also on the iron content of the food. In the above experiment a large excess of iron was administered in the readily assimilated ferrous ($Fe^{++}$) form, and this fact undoubtedly enhanced its absorption. The possibility cannot be overlooked

that excess iron is absorbed into the blood stream by simple diffusion through the mucosal cell without being retarded in its passage by the ferritin-apoferritin system.

The practical application of these results is clear.

Anaemia markedly increases the absorption of iron from the diet. If the anaemia is caused by a deficiency of iron—due to either (1) loss of iron through haemorrhage, or (2) deficiency of iron in the diet, or (3) malabsorption of dietary iron—it is reasonable to correct the deficiency by iron therapy. In all other forms of anaemia, iron therapy is contraindicated because iron is not excreted and the added iron, if given therapeutically, merely piles up in the iron stores, perhaps leading to early haemosiderosis if repeated transfusions are necessary in the treatment of the anaemia.

## SUMMARY.

The amount of storage iron in the liver and the spleen of rats was increased after prolonged treatment with phenylhydrazine. Under the conditions of the experiment the increased storage of iron could be attributed only to increased absorption of dietary iron. Increased iron absorption was also found in rats fed supplementary dietary iron.

The significance of these findings is discussed in the light of current views regarding the regulation of iron absorption.

*Acknowledgment.* The author wishes to acknowledge the technical assistance of Miss Marjorie Powell.

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# THE METABOLISM OF OYSTER SPERMATOZOA

## V. THE EFFECT OF GLYCINE

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*(Accepted for publication 19th April, 1954.)*

The beneficial effect of amino acids in prolonging the functional life of suspensions of spermatozoa has been established for sea-urchin (Tyler, 1950; Tyler and Atkinson, 1950), fowl (Lorenz and Tyler, 1951) and bull sperm (Tyler and Tanabe, 1952). The initial work gave no evidence for a metabolic utilization of the amino acid, although increases in oxygen uptake over a prolonged period of time were found in the case of sea-urchin sperm (Tyler and Rothschild, 1951) and fowl sperm (Lorenz and Tyler, 1951). It was concluded that amino acids enabled the sperm to utilize their endogenous reserves more efficiently. However, it is now suggested (Tyler, 1953) that the amino acids may act by removing toxic heavy metals as chelation complexes from the sea-water or other media in which the sperm are usually suspended. Artificial chelating agents were found to prolong the motile life and fertilizing capacity of suspensions of spermatozoa in a manner similar to amino acids. The cells also survived for a considerably longer period in artificial sea-water than in natural sea-water. In these highly purified salt solutions the beneficial effects of chelating agents were much reduced.

In this paper the effect of glycine on oyster spermatozoa is reported. This preliminary work shows the beneficial effect of glycine on sperm motility, and points towards a metabolic utilization of the amino acid. The significance of the metal-chelating properties of glycine will be discussed in a following paper.

### Materials and Methods.

Suspensions of spermatozoa were prepared according to Humphrey (1950). All solutions were made in sea-water and brought to pH 7·5–7·7, the optimal pH for oyster sperm metabolism. Solutions of oxalacetate, *cis*- and *trans*-aconitate and α-ketoglutarate were made up just before the addition of the sperm suspension in each experiment.

Total nitrogen was estimated by combustion with $H_2SO_4$ and $H_2O_2$, displacement of ammonia from a suitable aliquot into 0·02 N $H_2SO_4$ in a Conway diffusion chamber (to avoid chloride interference) and subsequent Nesslerization. Metabolic ammonia production was estimated by diffusion into $H_2SO_4$ after displacement from the experimental mixture with saturated $K_2CO_3$, followed by Nesslerization (Conway, 1947). Lactic acid was estimated according to the method of Barker and Summerson (1941).

Phase-contrast microscopy under highpower was used in all assessments of sperm motility, which was scored according to the notation of Emmens (1947).

[1]Working under a full time grant from the Research Committee, University of Sydney.

## RESULTS.

*Motility.*

The effect of glycine on sperm motility was tested at final concentrations of 0·001, 0·01, 0·05 and 0·1 M, with relatively dilute and relatively concentrated sperm suspensions. These contained approximately $5\times10^8$, $2\cdot5\times10^8$ and $0\cdot5\times10^8$ sperm/ml. One ml. of the suspension was added to 0·1 ml. of glycine and 0·05 ml. of 0·2 M phosphate buffer (pH 7·7) in test tubes for the aerobic tests, or Thunberg tubes for the anaerobic tests.

Fig. 1 shows the results of a typical series of experiments under aerobic and anaerobic conditions, and demonstrates the increasing effect of glycine in the concentration range 0·001 to 0·5 M. In most cases still higher concentrations showed no further effect. If initially the motility of the control sperm was only fair (as under anaerobic conditions), glycine at all concentrations increased the motility for about 2–3 hours, after which a gradual decline occurred. The sperm in glycine always remained distinctly more active than the sperm in the controls. If, however, the sperm were initially intensely motile (as under aerobic conditions), glycine was found to maintain the sperm in this maximum state for several hours, after which a decrease in motility occurred. These same patterns of activity were obtained for each of the three sperm dilutions studied.

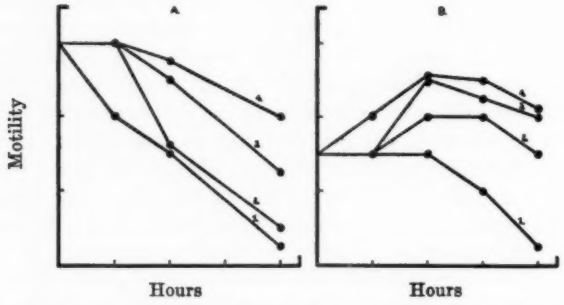


**Fig. 1.** The effect on the motility of oyster spermatozoa under Aerobic (A) and Anaerobic (B) conditions.

1=control
2=0·001 M glycine
3=0·01 M glycine
4=0·05 and 0·1 M glycine

*Respiration.*

The respiration of oyster sperm was studied over the same range of glycine concentrations as was used for the motility experiments. One ml. of sperm suspension ($5\times10^8$/ml.) was added to manometer flasks containing 0·01 ml. glycine (or sea-water), and 0·05 ml. 0·2 M phosphate pH 7·7. It was found

that glycine had a small but definite effect on respiration at a concentration of 0·01 M. Over a two-hour experimental period there was a mean increase of 10 p.c. (in 25 experiments) with a standard error of 2·9. In similar short-term experiments glycine had no significant effect on the $O_2$ uptake at a concentration of 0·001, 0·05 or 0·1 M.

*Ammonia formation.*

The amount of ammonia formed by sperm suspensions respiring endogenously is very small (about 0·5 $\mu$g./$10^8$ sperm/hour). When glycine was present at a concentration of 0·001 or 0·01 M, no increase in ammonia was detected under aerobic or anaerobic conditions. Increases of 15 p.c. (±4·5) and 30 p.c. (±5·0) were detected at glycine concentrations of 0·05 and 0·1 M, respectively, over a 2-hour period in air or nitrogen.

The effects of the ammonium ion on respiration and motility were tested at concentrations of $10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ M $NH_4Cl$. A 20–40 p.c. inhibition of respiration and a decrease in motility were found at a concentration of $10^{-3}$ M, but the lower concentrations tested showed no significant effects. It is thus apparent that the ammonia produced endogenously, or in the presence of glycine, had no beneficial effect on the survival of the spermatozoa.

On the other hand, Tyler and Atkinson (1950) suggested that low concentrations of ammonia had a favourable effect on sea-urchin sperm.

*Respiratory inhibitors.*

The ability of glycine to counteract or augment the effects of a number of respiratory inhibitors on sperm motility and respiration was studied. Azide, cyanide, iodoacetate, fluoride and *trans*-aconitate decreased the endogenous $O_2$ uptake of oyster sperm (Humphrey, 1950), whereas increases were usually observed with 0·01 M malonate, arsenate and selenite. However, the effects observed with the latter group were variable. Glycine (0·01 M) did not relieve the respiratory inhibition due to cyanide, azide, iodoacetate and fluoride, and caused a further 10 p.c. inhibition in the presence of *trans*-aconitate. Over a 2-hour period glycine increased the $O_2$ consumption by 23 p.c. in the presence of malonate, 18 p.c. with arsenate and 15 p.c. with selenite. A beneficial effect on sperm motility in the presence of the inhibitors was also demonstrated. Thus, at the end of the experimental period, sperm motility in the presence of the inhibitors alone was usually at a low level, but when glycine was present, activity was maintained.

Increases of 10–40 p.c. in the ammonia production were detected with malonate and arsenate (but not selenite) when glycine was present.

*Respiratory intermediates.*

The effect of glycine in promoting the oxidation of a number of respiratory intermediates by sperm suspensions was examined, using substrates at a final

concentration of 0·01 M. Previously, significant increases in $O_2$ uptake were found to result from the addition of oxalacetate, α-ketoglutarate, *iso*-citrate, pyruvate and sometimes malate (Humphrey and Jeffrey, 1954). Various other compounds were ineffective due to permeability factors. In the presence of oxalacetate, glycine caused a further increase of 29 p.c. (±4·5) in the $O_2$ uptake, and slight increases in ammonia formation were observed in half the experiments. With no other substrate (pyruvate, fumarate, malate, succinate, citrate, formate, acetate, propionate, butyrate, lactate, *cis*-aconitate and α-ketoglutarate) did glycine react similarly.

*Dehydrogenase activity.*

Methylene blue, at a final concentration of 0·0016 M, was used to test for the presence of glycine dehydrogenase activity in sperm suspension. Normally, this concentration was reduced by $5\times10^8$ sperm in about 40 minutes under anaerobic conditions. In 5 out of 10 experiments glycine was found to reduce the time for complete decolourization of the methylene blue by 40–50 p.c. On a number of occasions the activity of glycine and methylene blue was tested in the presence of 0·01 M malonate, arsenate and selenite. Glycine decreased the decolourization time by about 10 p.c. in the presence of these compounds.

*Glycolysis.*

Oyster spermatozoa accumulate very slight amounts of lactic acid under both aerobic and anaerobic conditions—0·2 μg. lactic acid/$10^8$ sperm/hour in the absence of added substrate. Glycolysis was re-examined in the presence of glycine which was added together with 0·01 M glucose, fructose, glucose-1-phosphate, glucose-6-phosphate, or fructose 1:6-diphosphate. As was previously found, fructose-diphosphate was the only substrate which gave rise to appreciable quantities of lactic acid. Glycine had no effect on lactic acid formation in the presence of any of these compounds.

## DISCUSSION.

Tyler and co-workers have established the beneficial effects of glycine and a variety of other amino acids and peptides on the viability of sea-urchin, fowl and bull spermatozoa. With sea-urchin sperm the effect on motility was even more marked under anaerobic than aerobic conditions (Tyler and Rothschild, 1951). Observations of the motility of oyster spermatozoa establish yet another species whose functional life span is greatly increased in the presence of glycine. This amino acid enables the sperm to exist in the absence of $O_2$ in a highly active state, whereas normally the cells soon lose motility under these conditions. Invertebrate sperm can remain alive, although motionless, for considerable periods of time in the absence of $O_2$ (Harvey, 1930). This indicates the existence of a metabolic system capable of functioning anaerobically. Glycine may enable the sperm to use this source of energy for motility.

Tyler and Rothschild (1951) used long term experiments to study the respiration of sea-urchin sperm in the presence of glycine. An initial lowering of $O_2$ uptake was obtained on the addition of glycine at final concentrations of 0·05 and 0·1 M, whereas lower concentrations (0·01 and 0·002 M) caused a temporary rise. This increase was interpreted as a dilution effect, which was abolished at higher concentrations of glycine. After two hours the rate of respiration of sperm in glycine was slightly greater than the controls, although at this time a net increase in $O_2$ consumption was not usually evident at all concentrations of glycine. However, over a 9–18-hour period, the total $O_2$ uptake in the amino-acid solutions was distinctly higher than the controls. A similar increase in respiration, correlating roughly with the increased duration of motility, has also been established for fowl sperm (Lorenz and Tyler, 1951). With oyster sperm an increase in respiration in the presence of glycine was successfully demonstrated over a 2-hour period. The experimental procedure which was employed excludes the possibility of the increase in $O_2$ uptake being due to dilution effects.

By the use of respiratory inhibitors, further effects of glycine on the metabolism of oyster sperm were demonstrated. In the presence of malonate and arsenate, two inhibitors which reduce the efficiency of oxidative phosphorylation, glycine increased the motility and respiration of the sperm. The demonstrated effects of glycine in the presence of the inhibitors may have been modified by their slowness of penetration through the sperm membrane. Marked variation was shown in the permeability of the cell to malonate (Humphrey and Jeffrey, 1954).

The results suggest that glycine is utilized for energy purposes when the normal efficiency of oxidative processes is decreased. This is supported by the methylene-blue experiments, which indicate the probable presence of glycine-dehydrogenase activity. Furthermore, the small but consistently observed increases in ammonia formation which result from the addition of glycine, together with the significant increases in $O_2$ uptake caused by glycine in the presence of oxalacetate, point to the existence of a pathway by which glycine can enter the metabolism of the cell. Previous studies on oyster sperm (Humphrey, 1950; Humphrey and Jeffrey, 1954) provide evidence for the existence in these cells of many of the steps of the citric-acid cycle, and it seems possible that glycine can enter this series of reactions by giving rise to $C_2$ units capable of condensation with oxalacetate.

As the results at present point only to the existence of a glycine pathway, it is not possible to state to what extent glycine may be utilized by these reactions under normal conditions. Even if this does play an appreciable part in the glycine effect of oyster sperm, it certainly provides no explanation for the beneficial effects of a wide variety of other amino acids on the motility of spermatozoa.

In conclusion, this work establishes a number of similarities in the effects of glycine on oyster sperm and sea-urchin sperm. These include the highly beneficial effect on the aerobic and anaerobic survival of the sperm cell, the small but definite increase in respiration which is accompanied by very small increases in ammonia formation, and the lack of any significant effect on aerobic or anaerobic glycolysis. The point of difference lies in the apparent absence of the metabolic pathway for glycine utilization in sea-urchin sperm, since Tyler and Rothschild (1951) were unable to find any production of isotopic $CO_2$ from carboxyl-labelled glycine.

## SUMMARY.

Glycine, in the concentration range 0·001 to 0·05 M was highly beneficial in prolonging the functional life-span of oyster spermatozoa under aerobic and anaerobic conditions.

At a concentration of 0·01 M, glycine increased both the endogenous respiration of sperm suspensions, and the respiration and motility in the presence of malonate, arsenate and selenite (but not cyanide, azide, iodoacetate, *trans*-aconitate or fluoride).

In the presence of oxalacetate, glycine caused significant increases in $O_2$ uptake. No similar effect was shown with pyruvate, fumarate, malate, succinate, citrate, formate, acetate, propionate, butyrate, lactate, *cis*-aconitate and α-ketoglutarate.

Glycine had no effect on the aerobic or anaerobic production of lactic acid.

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# THE METABOLISM OF OYSTER SPERMATOZOA

## VI. THE EFFECT OF METALS

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The use of chelating agents has provided indirect evidence that metals may play an important rôle in determining the length of survival of the sperm cell. It has previously been shown that the respiration of spermatozoa increases on dilution (Gray, 1928; Humphrey, 1950). Tyler and Rothschild (1951) found that the initial outburst of oxidative activity which occurs on dilution of spermatozoa was abolished by high (0·05–0·1 M) but not by low (0·01–0·001 M) concentrations of glycine. As copper and zinc ions in small quantities were responsible for the dilution effect of sea-urchin sperm (Rothschild and Tuft, 1950; Rothschild, 1950), the effect of glycine was interpreted as a removal of these ions from the sea-water in which the sperm was suspended. However, a toxic rôle for heavy metals in sperm metabolism was implied from the experiments of Tyler (1953) with chelating agents. The degree of extension of the life-span with ethylene diamine tetra-acetate and diethyldithiocarbamate was of the same order as with a number of amino acids, and from this it was concluded that both types of compounds were acting in the same capacity as metal chelators. Differences in the optimal concentration of these agents correlated roughly with differences in the dissociation constants of the metal-chelate complexes.

The effect of copper, zinc and cadmium on the respiration and motility of oyster spermatozoa has been studied in the present work. Also, the importance of the metal-binding capacity of glycine was investigated under these conditions.

### Methods.

Sperm suspensions were prepared according to Humphrey (1950). $CuSO_4$ and $ZnSO_4$ were of A.R. quality; $CdCl_2$ was C.P. (May and Baker). Solutions of the required concentration were made up in sea-water, and adjusted to pH 7·5–7·7 where necessary. Phosphate buffer was omitted from the metabolic experiments, because of removal of the metals as insoluble phosphates. At the end of each experiment the pH of the sperm suspension was still in the optimal range (7·2–7·5).

Potentiometric titrations, in carbonate-free triple-distilled water and in sea-water, were performed according to the method of Albert (1950). Glycine was used at a concentration of $5\times10^{-3}$ M, with metals at a concentration of $2{\cdot}5\times10^{-3}$ M and $10^{-4}$ M, in a final volume of 50 ml. During titration in the distilled water medium, mixing was effected by bubbling a

[1]Working under a full time grant from the Research Committee, University of Sydney.

fine stream of $CO_2$-free nitrogen for 1 minute between readings. Mixing with a mechanical stirrer was necessary for the sea-water experiments, as the nitrogen rapidly removed $CO_2$ from the sea-water, with consequent rise in pH. Titrations were carried out with 0·098 N carbonate-free NaOH, using a Cambridge glass electrode.

## RESULTS.

### *Respiration.*

The respiration of oyster spermatozoa was studied in the presence of $CuSO_4$, $ZnSO_4$ and $CdCl_2$. The final concentrations of added metal salts were $10^{-5}$, $10^{-4}$, $10^{-3}$ and $5\times10^{-3}$ M. In order to allow immediate chelation to take place, glycine was mixed with the metal salt before the addition of the sperm suspension. The results of a typical series of experiments are shown in Table 1.

TABLE 1.

*Respiration of oyster spermatozoa in the presence of $Cu^{++}$, $Zn^{++}$ and $Cd^{++}$.*

1 ml. sperm suspension ($5\times10^8$/ml.) +0·1 ml. 0·1 M glycine (or sea-water) +0·1 ml. metal salt were present in each flask. Results are expressed as p.c. of endogenous $O_2$ uptake over a 2-hour period.

| Final molar concentration of metal* | Cu | Cu glycine | Zn | Zn glycine | Cd | Cd glycine |
|---|---|---|---|---|---|---|
| $10^{-5}$ | 110 | 112 | 85 | 85 | 120 | 125 |
| $10^{-4}$ | 115 | 120 | 69 | 70 | 138 | 143 |
| $10^{-3}$ | 100 | 105 | 32 | 38 | 161 | 159 |
| $5\times10^{-3}$ | 22 | 22 | 28 | 28 | 109 | 106 |
| $10^{-2}$ | — | — | — | — | 25 | 21 |

* The endogenous respiration of 1 ml. sperm suspension is taken to be 100.

An increase in the respiration of oyster spermatozoa resulted from the addition of low concentrations of copper and cadmium. High concentrations of these metals caused an inhibition. However, decreases in the respiration were obtained at all concentrations of zinc. When glycine was present, no alteration in the effects of copper, zinc and cadmium on respiration was demonstrated. The usual small increases due to 0·01 M glycine were probably lost in the greater percentage changes which took place in the metal experiments.

A difference in the behaviour of oyster sperm and sea-urchin sperm to zinc salts is apparent from the work of Rothschild and Tuft (1950), who found that zinc as well as copper stimulated the respiration of sea-urchin sperm at low concentrations. However, the behaviour of the two species to cadmium is similar, although smaller effects were obtained with oyster sperm. Thus Barron *et al.* (1948), using sea-urchin sperm, reported increases of 140 p.c. and 51 p.c. in the presence of $10^{-4}$ and $10^{-3}$ M $CdCl_2$, respectively, over a 2-hour period.

*Motility.*

The motility of oyster spermatozoa ($5\times10^8$/ml.) was studied in the presence of metal ions and glycine over a similar range of concentrations as was used in the respiration experiments. Copper and zinc at a final concentration of $10^{-6}$ M activated the spermatozoa over a 4-hour period. No effect was shown at a concentration of $10^{-5}$ and $10^{-4}$ M. At higher concentrations ($10^{-3}$ M) motility was lost within one hour. Glycine did not alter the beneficial effects of low concentrations of these metals. In the range $10^{-5}$ to $10^{-4}$ M, where the metal had no effect, the usual increase in motility due to 0·01 M glycine was apparent. Glycine protected the sperm against the harmful effects of high concentrations ($10^{-3}$ M) of copper, but not of zinc.

Cadmium (from $10^{-6}$ to $10^{-3}$ M) increased the motility of oyster spermatozoa. The marked effect obtained with $10^{-3}$ M cadmium was shown over a prolonged period, the sperm retaining high activity for several hours after motility had ceased in both the sea-water and glycine controls. Glycine did not alter the beneficial effects of cadmium at any concentration of the metal.

The results show that the activating effect of low concentrations of cadmium on sperm motility is associated with increases in the respiration. Where the addition of copper and zinc salts adversely affected motility, appreciable inhibition of respiration was always obtained. No effect of motility was demonstrated with these metals at levels ($10^{-5}$ and $10^{-4}$ M) where only small respiratory changes occurred.

*Formation of chelation complexes.*

Chelation between metal ions and amino acids can usually be detected by displacement of the pH titration curves, and delay in the precipitation of the metallic hydroxide. The curves obtained for the titration of glycine ($5\times10^{-3}$ M) with copper, zinc and cadmium ($2{\cdot}5\times10^{-3}$ M) in sea-water are shown in Fig. 1. From these data, it is possible to calculate the quantities of free and bound metal ions present, on the basis of the theoretical treatment of Maley and Mellor (1949, a and b). Thus, in the sea-water system containing copper ($2{\cdot}5\times10^{-3}$ M) and glycine ($5{\cdot}0\times10^{-3}$ M) the proportions of reactants and products at pH 7·7 are as follows:

| | |
|---|---|
| $Cu^{++}$ | $1{\cdot}202\times10^{-6}$ M. |
| Cu-Glycine$_1$ | $0{\cdot}9\times10^{-3}$ M. |
| Cu-Glycine$_2$ | $1{\cdot}6\times10^{-3}$ M. |
| Glycine | $0{\cdot}9\times10^{-3}$ M. |

Data from the potentiometric titrations indicate that chelation takes place in sea-water between $5{\cdot}0\times10^{-3}$ M glycine and $2{\cdot}5\times10^{-3}$ M copper and cadmium. In the case of zinc it is not possible to determine the extent of complex formation, in view of precipitation phenomena which occur at pH 7·7. This is probably due to the formation of hydroxy-zinc complexes.

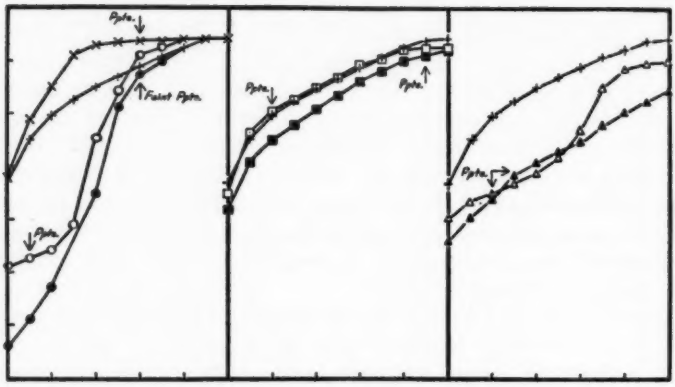

**Fig. 1.** Chelation-titration curves of metal-glycine complexes in sea-water. Glycine ($5\times10^{-3}$ M) +metal ($2{\cdot}5\times10^{-3}$ M) in 50 ml. sea-water.

Abscissa=Ml. NaOH (0·098 N)
Orindate=pH.
×——×=seawater
+——+=glycine
○——○=Cu
●——●=Cu+glycine
□——□=Cd
■——■=Cd+glycine
△——△=Zn
▲——▲=Zn+glycine

As high concentrations of copper and cadium chelate with glycine in sea-water, binding of lower concentrations of metals (under similar experimental conditions) must also occur. However, only in distilled water was complex formation with $10^{-4}$ M metals and $5\times10^{-3}$ M glycine readily demonstrated. The slight buffering effect due to the $CO_2$ system in sea-water, and the high salt concentration, probably masked pH changes.

## DISCUSSION.

Copper (MacLeod, 1951) and cadmium (Barron *et al.*, 1948) are thought to act in sperm metabolism by combining with sulphydryl groups to form mercaptides. Barron has suggested that at low concentrations the metals react with soluble sulphydryl regulators in the cell, thus producing an increase in respiration. At higher concentrations, the combination of metals with fixed enzymic —SH groups results in an inhibition.

The potentiometric titrations indicate that glycine forms chelation complexes with copper and cadmium in sea-water. However, in the presence of spermatozoa, glycine did not alter the characteristic effects of the metal ions on respiration and motility. If the physiological effect of copper and cadmium is due to their affinity for sulphydryl groups, a possible explanation of the results is that the stability of the metal-S bond is greater than that of the metal-glycine bond.

Thus, in the presence of the spermatozoa, copper and cadmium would be displaced from combination with glycine, and the characteristic effects of these metals on respiration and motility obtained.

Chelation between glycine and zinc in sea-water at pH 7·7 is probably limited by the formation of hydroxy-zinc complexes. This may explain why the zinc inhibition of sperm respiration and its effects on motility were not altered in the presence of glycine. In contrast to copper and cadmium, no function has been postulated for zinc in sperm metabolism.

The prolongation of the functional life-span of sperm suspensions by glycine has been ascribed to the inactivation of toxic metals present in suspending media (Tyler, 1953). Copper and zinc were suggested as possible metals involved. These occur in sea-water at concentrations of $10^{-7}$ and $10^{-8}$ M (Barnes and Rothschild, 1950; Atkins, 1936). Cadmium, which has been identified in marine organisms, but not in sea-water, may occur at even lower levels (Harvey, 1945). The present work demonstrates that very low concentrations of copper, zinc and cadmium are beneficial to the motility of oyster spermatozoa, and copper and cadmium also increase the respiration. Glycine did not alter the effects of these metals at any tested concentration ($10^{-6}$ to $10^{-3}$ M). This implies, that under similar experimental conditions, glycine would not remove the low concentrations expected in sea-water, or in other artificial suspending media. However, this work does not rule out the possibility that other metals present in sea-water with higher stability constants may be involved.

In conclusion, this preliminary work has demonstrated a metabolic effect of glycine on oyster spermatozoa (Jeffrey, 1954). Also, the results offer no support for the hypothesis that the beneficial effect of glycine is due to the removal of small quantities of copper, zinc or cadmium from suspending media.

## SUMMARY.

Copper, zinc and cadmium at low concentrations were beneficial to the motility of oyster spermatozoa. Increases in respiration were obtained with low concentrations of copper and cadmium, whereas high concentrations caused marked inhibition. Zinc decreased the respiration of oyster sperm at all concentrations.

Potentiometric titrations demonstrated the formation of glycine-chelate complexes with copper and cadmium in sea-water at the physiological pH (7·7). Chelation probably occurs only to a small extent with zinc due to interfering precipitation effects.

Glycine did not alter the characteristic effects of copper, zinc or cadmium on the respiration and motility of oyster spermatozoa.

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# COMMERCIAL DRIED EGG ALBUMEN AS A PROTEIN SOURCE FOR THE GROWING RAT

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*(Accepted for publication 12th July, 1954.)*

The purpose of the investigation was to determine by comparatively long-term experiments the biological value of commercial dried egg albumen when ingested as the major protein source in the dietary of the growing rat. Dried egg albumen of this type could be a useful source of protein, particularly when a low, or controlled, fat intake was required for experimental purposes, provided the known nutrient antagonists present did not exert any appreciable influence. Work reported up to the present time has been short-term studies of the effect on the growth of experimental animals by Klose, Hill and Fevold (1950), and nitrogen-balance experiments on man by Scudamore, Morey, Consolazio, Berryman, Gordon, Lightbody and Fevold (1949). Himsworth (1950) reports that a proportion of rats fed soy bean develop necrotic lesions of the liver, causing death, and that this is due to the presence of an active proteolytic enzyme inhibitor in soy bean, also found in other legumes as reviewed by Almquist (1951). In demonstrating this effect in class work in this Department, it has been noted that no abnormalities of growth or health of the rats could be detected until a very short period before death from the hepatic lesions.

From these observations it can be concluded that short-term growth studies may not reveal the effects of active nutrient antagonists such as the trypsin inhibitor of soy bean, or those present in egg white.

## Preliminary Experiment.

A preliminary experiment was conducted with a small number of weanling albino rats, in three groups with equal number of sexes in each group. The dietaries, which were fed *ad lib.*, contained protein, at a level of 15 p.c. by weight, as the variant. The proteins selected were: Casein as control, commercial dried egg white, and casein and dried egg white in equal proportions. The casein in each case was supplemented with the amino acids cystine and methionine to reach levels equivalent to those normally found in egg white as indicated by the Tables of amino acid content of proteins reported by Block and Bolling (1951).

Details of the three dietaries are given below:

Group I. Control. Casein, supplemented with cystine and methionine, 15 p.c.

Group II. Dried egg albumen, 15 p.c.

Group III. Dried egg albumen 7·5 p.c., and casein, supplemented, 7·5 p.c.

The remaining basic constituents were identical for all dietaries and consisted of the

following: Cornflour, 63 p.c.; lard, 10 p.c.; dried yeast, 6 p.c.; salt mixture, 4 p.c.; cod liver oil, 2 p.c.

Although the dried yeast would contribute some protein, this was disregarded as the amount would be identical for all groups and the contribution not appreciable.

Bi-weekly weighings were made of each rat, and the total group dietary intakes also estimated once weekly.

After five weeks a male and a female rat from each group was killed by ether and examined and the remainder of the rats were killed at the conclusion of the thirteenth week. After *post-mortem* examination, the livers, kidneys and spleens were weighed and histological examination made of the livers and kidneys. In addition, determinations were made of moisture content and the total cholesterol content of the livers of the rats killed at the thirteenth week period.

The weight gain, food consumption and weight of organs showed little of any significance. Of importance was the finding that all members of Group II showed some degree of fatty infiltration of the liver, ranging from a slight to a gross effect.

An increased average total cholesterol content of the livers, particularly in respect to the male rats, also suggested metabolic abnormality. Generally higher levels of normality of total cholesterol in the liver were found in the female rats of all groups when compared with the results for the normal male values.

Abnormality in respect to weight and macroscopic appearance of certain livers was confirmed by the histological examinations which showed gross fatty infiltration.

These findings suggested the need for further investigation for confirmation of the results with a larger group of animals. The details of this main investigation are reported below.

## Main Investigation.

## EXPERIMENTAL METHOD.

Weanling male albino rats were selected in view of the findings of the preliminary experiment. Three groups were arranged, of equivalent average weight, with the following numbers per group: Sixteen in Group I, sixteen in Group II, and twelve in Group III, a total of 44 rats. Unfortunately, it was not possible to obtain the requisite number of male rats of exactly comparable age and, as a result, the initial individual weights at the commencement of the test showed considerable variation, thus reducing to some extent the value of possible findings in connection with rate and extent of growth. This, however, in the preliminary experiment had been shown to have little importance. The rats were maintained on the following dietaries, fed *ad lib.*, for a period of approximately fifteen weeks.

Group I. Control. Casein, supplemented with amino acids methionine and cystine to reach levels comparable with egg white, 15 p.c.

Group II. Dried egg albumen, 15 p.c.

Group III. Dried egg albumen 7·5 p.c., and casein, supplemented, 7·5 p.c. Basic components similar for all dietaries were cornflour, lard, dried yeast, cod liver oil and salt mixture in the proportion described for the preliminary experiment.

Bi-weekly weighings were made of all rats and weekly record also made of the group food intakes.

From the fourth week of the experiment, symptoms occurred suggestive of biotin deficiency, and therefore it was decided to administer biotin to half the members of all groups in curative doses of 120 $\mu$g. to each member per week, an amount suggested for oral administration by the work of György and Rose (1940) and by Handler (1946). This was given in

three doses each week from the tenth week, by pipette for the first week of administration, but for the remainder of the test given in water bottles, one bottle with twice the estimated dose for the two rats housed in each cage.

During the fifteenth and sixteenth week after commencement of the test, when growth was almost static, all the rats were killed by means of coal gas. After post-mortem examination, the livers, kidneys and spleens were weighed. Estimations were then made of the moisture content, total cholesterol and total lipid content of the liver of each rat. Total cholesterol was determined by a slight modification of the method of Sackett (1925), also cited in King (1951). Total fat was determined using aliquots of the extract made for cholesterol estimation, and by titration as the fatty acids, and the conversion of the result into terms of total fat, as described by Van der Kamer, Bokkel Huinink and Weyer (1949).

## RESULTS AND CONCLUSIONS.

At *post-mortem* examination all animals appeared in healthy condition. Organs were normal in appearance with the exception of pale or blotchy livers, suggestive of fatty infiltration, of certain members of Groups II and III. A few animals in these groups showed some slight abnormalities of, or of the residues within, the alimentary tract, correlating with a recent incidence of a relaxed condition of the bowel.

Study of the results of analyses for individuals revealed that in Group II (egg albumen) six of the sixteen rats showed definite abnormalities, particularly in respect to total cholesterol and total fat in the liver, which were at very high levels. Of these six rats, four were grossly abnormal in this respect, correlating with macroscopic abnormalities of the livers noted at *post-mortem*. The remaining two showed levels for these two components well above the upper limits of normality, but macroscopic examination of the livers did not show conclusive evidence of abnormality. All other members of this group showed normal findings.

In Group III (mixed proteins) four of the twelve rats showed high levels of total fat and total cholesterol in the liver, all other members being within the normal range. No abnormality was observed in any member of Group I (casein), the control. Liver weight was not of importance, although, for each of the abnormals within both groups, irrespective of body weight, this was in the upper range of weights recorded, particularly so when expressed as a percentage of body weight. One rat in Group II, found to have a grossly elevated value for liver total cholesterol, had shown *post-mortem* a typical "large fatty" liver. In the case of the controls and normal members of the two groups, liver, kidney and spleen weights followed the pattern of body weight. Moisture content of the livers of the abnormals was generally in, or somewhat below, the lower levels for the normals, which finding suggests the replacement of active functioning tissue with lipid material.

Body weight gain in the abnormals did not appear significant, either calculated as total weight gain or as percentage gain on initial body weight. Generally,

for all members, this correlated with initial body weight which latter could be taken to indicate approximately age from birth at the commencement of the test, that is, those of low initial weight, presumably younger in age, gained a greater amount in body weight throughout the test period which continued until the usual flattening out of the growth curve as maturity was reached.

A minority of the abnormals had received a biotin supplement, but, owing to the small number of abnormal animals, little can be deduced from the result; a discussion concerning the effects of biotin administration is given later.

A summary of the results obtained is shown in Table 1, in which are presented the individual results for those members classified as abnormal following *post-mortem* and biochemical investigation in both Groups I and II, and the means and variances of the results for the normals within these groups, and for the control group.

TABLE 1.

*Results of analyses of rat livers.*

| Group | No. of animals | Total cholesterol in liver mgm./gm. dry wt. liver | Percentage moisture in liver | Percentage total fat in liver |
|---|---|---|---|---|
| GROUP I | 16 | | | |
| Controls-Casein | | | | |
| S.D. | | 9·31 | 1·57 | 1·60 |
| Mean | | 42·82 | 69·57 | 8·78 |
| GROUP II | 16 | | | |
| Egg albumen | | | | |
| *Normals* at P.M. | 10 | | | |
| S.D. | | 6·70 | 1·45 | 1·66 |
| Mean | | 45·42 | 69·53 | 8·59 |
| *Abnormals* at P.M. | 6 | | | |
| II.2 (+biotin) | | 243·3 | 65·6 | 32·5 |
| II.5 | | 198·0 | 63·8 | 20·0 |
| II.6 | | 298·4 | 62·1 | 16·8 |
| II.12 (+biotin) | | 228·0 | 56·1 | 19·0 |
| II.15 | | 77·4 | 69·3 | 10·1 |
| II.16 | | 80·1 | 68·1 | 8·5 |
| GROUP III | 12 | | | |
| Mixed proteins | | | | |
| *Normals* at P.M. | 8 | | | |
| S.D. | | 9·07 | 1·15 | 1·03 |
| Mean | | 41·48 | 68·30 | 9·02 |
| *Abnormals* at P.M. | 4 | | | |
| III.6 (+biotin) | | 77·1 | 68·2 | 8·8 |
| III.8 | | 85·1 | 69·0 | 12·0 |
| III.9 | | 89·5 | 66·9 | 15·1 |
| III.12 | | 62·4 | 70·5 | — |

Study of this Table shows clearly the definite abnormality of the findings, that is, the high levels of total cholesterol and total fat in the livers, for those rats classed as abnormal at *post-mortem* in both groups, but particularly in Group II, when compared with those of the control group, or normals at *post-mortem* within the groups.

Liver total cholesterol has been cited by Abell and Beveridge (1951) as of greater significance in indicating certain metabolic disorders or impending function or organic damage to the liver than is total fat in the liver. Normality is based not only on the findings for the control group, but also on levels for lipids in rat liver, reported by Güerring, Hurley and Morgan (1952).

The findings for the other individuals are reported as the means and variances for the normals within each experimental group. These results interpreted statistically indicate that for each quantity the maximum variance ratio is not significant when referred to Tables of F (Snedecor, 1938), so that there is no evidence that the variances change from group to group. Again for each quantity, an analysis of variance within and between groups (Snedecor, *op. cit.*) shows no significant differences between the means of the three groups. This suggests that the response to the egg albumen in the dietary is a matter of individual reaction as certain members only were affected, and these quite severely, while normal growth and metabolic functioning was maintained by the majority under similar conditions of dietary and environment. It is not possible at this stage to postulate possible reasons for such variations between individuals as there did not appear to be any common factors or characteristics suggesting susceptibility of the particular individuals noted during the course of the experiment.

These effects produced are assumed to result mainly from the active trypsin inhibitor, identified as ovomucoid by Lineweaver and Murray (1947), and reported present in dried egg white (Feeney, Ducay, Silva and MacDonnell, 1952). Such an inhibitor by interference with protein absorption results in an inadequate intake of amino acids, including the essential amino acids, and a deficiency of methionine and cystine and other lipotropic factors is known to produce disturbance of liver function resulting in the production of "fatty livers." The effect of the different dietaries on the abnormality rate is shown in Table 2.

TABLE 2.

*Effect of dietaries on abnormality rate.*

| | Casein only | Casein and egg albumen | Egg albumen only | Totals |
|---|---|---|---|---|
| **Normals** | 16 | 8 | 10 | 34 |
| **Abnormals** | 0 | 4 | 6 | 10 |
| **Totals** | 16 | 12 | 16 | 44 |

The analysis of this contingency Table by the method of Yates (1948) shows that there is a significant relationship between the amount of egg-source protein and the abnormality rate and that this relationship is linear.

This indicates that the ingestion of dried egg white is the factor responsible for the production of the above-mentioned symptoms of hepatic dysfunction, and this would preclude its use as a major protein source in experimental dietaries for studies concerned with rat growth.

*Other effects noted during the experiment.*

*Biotin deficiency.* At the period of maximum growth rate, and hence nutritive requirement, symptoms of biotin deficiency were noted in both groups receiving egg white in the dietary. This diagnosis was based on the fur loss, or alopecia, and a preliminary or accompanying porphyrin-like pigment in the fur, which latter could not be identified as a porphyrin by fluorescence. Confirmation of this diagnosis was given by the treatment with curative doses of biotin, which caused complete remission of the symptoms described. The severity of the condition appeared directly related to the proportion of egg white in the dietary. It can be concluded that a biotin deficiency had developed, and that this was created by the presence of avidin in the dried egg white, as has been described in the literature on this subject (Fraenkel-Conrat, Snell and Ducay, 1952).

The small number of abnormal animals concerned prevents conclusive analysis in regard to possible contribution of the biotin deficiency toward the hepatic abnormalities, but the results and observations do suggest that the relationship, if any, is of very minor importance.

*Gastrointestinal symptoms.* Intermittent attacks occurred, at approximately the same period as did the symptoms of biotin deficiency, of a mild diarrhoea which affected all members of Group III (mixed proteins), some members of Group II (egg albumen), and none of Group I (casein).

The condition was not of infective origin, and it is suggested that, by using dried yeast as the source, the B-complex vitamins, possibly specifically folic acid, were at a minimum level of intake for growth and then only when all other metabolic requirements were satisfied.

A possibility which might warrant further investigation is that the physical texture of the dried egg albumen may adversely affect the gastric mucosa.

## SUMMARY.

Commercial dried egg albumen, fed as the major protein source in the dietary of the growing rat, produced in some cases symptoms of hepatic or metabolic dysfunction. Fatty infiltration of the liver, as indicated by abnormally high levels of total cholesterol and total lipid, were found in ten of the twenty-eight rats fed this product.

Findings for the other members, compared with those for the controls, showed no statistical variation.

It is concluded that until such time as the predisposing factor, or factors, for such individual response have been determined, commercial dried egg white cannot be regarded as a satisfactory protein source in the dietary for the growing rat.

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# THE ANTIKETOGENIC ACTION OF SORBITOL IN RATS

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Using liver slices from starved rats, Edson (1936) showed that sorbitol and glycerol, but not other polyhydric alcohols, were particularly effective in suppressing endogenous formation of acetoacetic acid. Sorbitol decreased acetoacetic acid production by 75 p.c., whereas glucose and fructose caused smaller inhibitions.

Blakley (1951) confirmed these observations and showed that the first step in the metabolism of sorbitol is conversion to fructose, brought about by a diphosphopyridine nucleotide (DPN)-linked dehydrogenase (L-iditol dehydrogenase (Edson, 1953)). This enzyme is present in rat liver but absent from other organs except the kidney where activity is low. Intact liver tissue transforms fructose into glucose. Hence the main product of sorbitol oxidation released from perfused dog liver and from rat-liver slices is glucose, accompanied by small amounts of fructose (Embden and Griesbach, 1914; Blakley, 1951). Moreover, Stetten and Stetten (1951) have shown that $^{14}C$-labelled sorbitol is converted into liver glycogen by the normal rat and is excreted as glucose by the alloxan-diabetic rat.

In order to determine its quantitative importance, the antiketogenic effect of sorbitol has been studied in the intact rat and compared with that of other substances.

## EXPERIMENTAL.

Rats of the Wistar strain and piebald rats of the Otago Medical School colony were used. The rats were fed on a stock diet.

The animals were anaesthetized by intraperitoneal injection of sodium amytal (120 mg./kg.) and kept on an operating table thermostatically heated to 37° C. The trachea was exposed by a small longitudinal incision between the sternohyoid muscles and elevated sufficiently to permit the insertion of a fine glass rod dorsal to the trachea. The trachea was opened by a small incision between two cartilaginous rings. During an experiment the airway was kept clear by extracting mucus with filter paper and, if necessary, by suction with a pasteur pipette. The external jugular vein and the long saphenous vein were exposed and covered with saline-moistened pads of cotton wool. The animals were allowed

to "stabilize" at this stage for 0·5 to 0·75 hours after the administration of the anaesthetic. Consistent resting blood sugar values were obtained by taking this precaution. Serial blood samples were removed from the jugular vein, the needle (gauge 26) being inserted through the pectoralis major muscle to prevent bleeding from the needle puncture. The substance under investigation (dissolved in 1·0 ml. of water) was injected slowly (taking 5 min.) into the long saphenous vein. At the conclusion of an experiment the wounds were dusted with penicillin-lactose powder and closed with Michelle clips. After several weeks some animals were used again.

Wistar rats were made diabetic by injecting alloxan monohydrate (2 p.c. solution made up in acidulated water just before use) (Patterson, Lazarow and Levy, 1949) into the long saphenous vein under ether anaesthesia. The rats received 40 to 60 mg./kg., according to age (Charalampous and Hegsted, 1949). There were many deaths (40 p.c.) and few of the surviving animals remained diabetic for longer than 3 to 4 weeks. Piebald rats (6 months old) were rendered diabetic by injection of 40 and 45 mg. of alloxan/kg. for does and bucks respectively. Thirty to 36 hours later they received 3 to 4 units of protamine zinc insulin subcutaneously. Thereafter these rats received 3 units of protamine zinc insulin daily. Three days after the injection of alloxan the rats were given a second intravenous injection of alloxan (80 mg./kg.). Maintained with insulin, these animals appeared normal and gained weight, but when insulin was withheld they developed glycosuria and ketosis in 2 to 4 days. If left longer without insulin, they died in diabetic coma. About 30 p.c. of the rats died 2 to 3 days after the initial injection of alloxan. Brodsky, Nelson and Guest (1952) obtained ketotic rats in a similar manner. In starvation experiments rats received water *ad libitum* but no food.

Blood samples (about 0·200 ml.) were drawn into dry, cooled Tuberculin syringes, ejected into small corked and tared centrifuge tubes containing 2 ml. of ice-cold water and gently shaken until laked. The tubes were recorked, weighed and returned to the cold room pending analysis at the end of an experiment. Analytical results were calculated from the weight of blood.

### Analytical Methods.

Reducing sugar was determined titrimetrically or colorimetrically according to Somogyi (1945a, 1952) after deproteinization with $Ba(OH)_2$—$ZnSO_4$ (Somogyi, 1945b). The total volume of filtrate was 8·0 ml. of which 0·5 to 2·0 ml. were taken for the reducing sugar estimation, depending on the expected blood-sugar level.

Fructose was determined by Cole's modification of Roe's method (see Bacon and Bell, 1948). The colour intensity was estimated by measuring optical density at 483 $m\mu$.

Total ketone bodies were determined by the method of Greenberg and Lester (1944) and the results expressed as acetone (mg./100 ml. of blood). The 2, 4-dinitrophenylhydrazine was purified according to Lester and Greenberg (1948).

## RESULTS.

Normal values were established on blood withdrawn from anaesthetized normal rats. In 17 rats mean initial acetone bodies were found to be 1·32 mg. (standard deviation ±0·65 mg.) acetone/100 ml. blood. In 50 rats mean resting blood glucose was 90 mg. (standard deviation±7 mg.)/100 ml. blood. There was no detectable fructose. In well "stabilized" control animals the blood ketone bodies and glucose remained essentially constant over the 2-hour period of an experiment.

*Effect of sorbitol on phlorrhizin-induced ketonaemia in Wistar rats.* It is well known that the injection of phlorrhizin into experimental rats produces glycosuria and ketonuria (Nash, 1927). In the dog, feeding glucose or fructose considerably decreases the ketonuria (Wierzuchowski, 1925). The effect of sorbitol on phlorrhizin ketonaemia in Wistar rats is shown in Fig. 1a. Sorbitol temporarily increases the blood-sugar level and causes a reduction in the concentration of ketone bodies. A control animal which received 1·0 ml. of saline (Fig. 1b) did not show a decrease in ketonaemia.

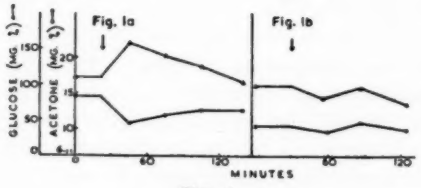


Fig. 1.

**Fig. 1. Effect of sorbitol on phlorrhizin ketosis. Rats injected intraperitoneally with 0·04 gm. of phlorrhizin in 0·8 ml. of olive oil and experiments performed 36 hours later. (a) Rat (240 gm.) received 0·18 gm. of sorbitol intravenously. (b) Rat (220 gm.) received 1·0 ml. of saline intravenously. Essentially the same antiketogenic effect was obtained in two other phlorrhizinized rats.**

Mannitol, which is not oxidized at a significant rate by rat liver, had no appreciable effect on the blood ketone-body level (experiments with two rats).

*Effect of sorbitol and fructose on ketonaemia in diabetic Wistar rats.* Despite 24 hours' starvation the ketonaemia is slight (1–2 mg. acetone/100 ml.) in diabetic Wistar rats exhibiting polyuria, glycosuria and diabetic cataracts. In experiments with four diabetic Wistar rats it was found that sorbitol and fructose reduced the already low ketone-body level by almost one half. After the injection of sorbitol a small amount of fructose was always detected in the blood, confirming the view that sorbitol is metabolized through fructose in the whole animal as well as in isolated liver tissue.

*Effect of sorbitol, glucose, glycerol and dulcitol on ketonaemia in starved piebald rats.* Rats of the piebald strain are noticeably fatter than those of the

Wistar strain and exhibit an appreciable ketonaemia during starvation. After 48 hours without food, blood ketone-body levels range from 8 to 15 mg. acetone/100 ml. The effects of sorbitol and glucose on the ketone-body levels of such starved rats are shown in Fig. 2. Both substances cause the removal of ketone bodies from the blood, and in both cases the blood ketone-body level is lowered by about 75 p.c. in one hour, but rises again as the effects of the injected substances diminish.

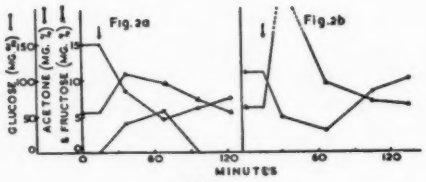


Fig. 2.

**Fig. 2.** Effect of sorbitol and of glucose on ketonaemia in piebald rats starved for two days. (a) Rat (210 gm.) received 0·25 gm. of sorbitol intravenously. (b) Rat (225 gm.) received 0·25 gm. of glucose intravenously. Similar figures were obtained with one other pair of starved piebald rats.

In the rat-liver-slice experiments of Edson (1936), glycerol was strongly antiketogenic. A similar effect was observed when glycerol was given by the intraperitoneal route. In one hour blood ketone bodies dropped from 7 to 2·7 mg. acetone/100 ml. after the injection of 1·25 ml. of 20 p.c. w/v glycerol solution. The same intravenous dose of glycerol produced a similar effect but proved too toxic to permit valid conclusions, as Deichmann (1940) has already indicated.

The intravenous injection of dulcitol in two rats produced no more effect than the injection of 1·0 ml. of normal saline (cf. Fig. 1). The experiments confirm the observations of Carr and Krantz (1934), who have shown that in the rat dulcitol does not affect the blood-sugar level, and they are consistent with the fact that dulcitol is not antiketogenic in liver slices from starved rats (Edson, 1936).

*Effect of sorbitol and fructose on ketonaemia in piebald rats rendered ketotic by feeding butter.* Alloxan-diabetic rats of the piebald strain do not become severely ketotic during starvation (acetone up to 10 mg./100 ml. of blood). Sorbitol reduces this ketosis effectively.

In an attempt to obtain severe ketosis, piebald rats which had been diabetic for several months (excreting 8–10 gm. of glucose/day), were fed a restricted carbohydrate diet containing excess butter for one week. This induced a marked ketosis and fatty infiltration of the liver. Both sorbitol and fructose lowered the blood ketone-body level slightly for a brief period after which it continued to

rise (Fig. 3). It would seem that butter-induced ketosis is resistant to the antiketogenic action of sorbitol and fructose, possibly due to the fatty condition of the liver.

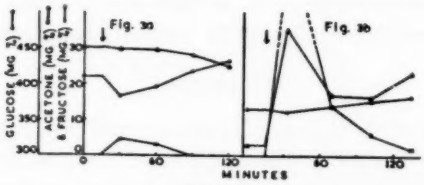


Fig. 3.

**Fig. 3. Effects of sorbitol and of fructose on butter-induced ketonaemia in diabetic piebald rats. Rats starved 48 hours before experiments. (a) Rat (245 gm.) received 0·25 gm. of sorbitol intravenously. (b) Rat (275 gm.) received 0·25 gm. of fructose intravenously.**

*Effect of sorbitol and fructose on ketonaemia of severely ketotic diabetic piebald rats.* In the experiments described above, attempts to induce severe diabetic ketosis similar to that in human beings by starving or high fat feeding of alloxan-diabetic rats, were unsuccessful. However, alloxan-diabetic rats of the piebald strain maintained on insulin almost invariably became ketotic, and eventually comatose, when insulin was withheld. In the following experiments the rats were semi-comatose. The severity of their condition is indicated by the fact that they died subsequently in spite of insulin and sorbitol treatment.

Two typical experiments are shown in Fig. 4. Sorbitol reduces the ketonaemia in both cases, but appears relatively more effective in the less severely ketotic animal. The drop in ketone bodies 75 min. after sorbitol injection is 9 and 27 mg. acetone/100 ml. blood, respectively. The blood-fructose level in Fig. 4b is high and remains high throughout the experimental period in contrast to the fructose level in previous experiments (Fig. 2a). This effect was observed in other severely ketotic diabetic rats treated with sorbitol. The livers of the semi-comatose rats used were heavily infiltrated with fat.

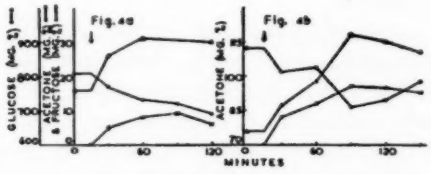


Fig. 4.

**Fig. 4. Effect of sorbitol on ketonaemia in diabetic piebald rats. Insulin was withheld for 3 days before experiment. Rats weighed 250 gm. and 310 gm. in experiments (a) and (b) respectively. Both received 0·25 gm. of sorbitol intravenously. Similar results were obtained with three other pairs of severely ketotic rats having the same degree of hyperglycaemia and ketosis.**

The antiketogenic effect of fructose is similar to that of sorbitol in these animals. Again fructose was slowly removed from the blood. In control experiments with ketotic diabetic rats (2) injected with normal saline, both blood ketone-body and glucose levels rose at a steady rate, and the intravenous administration of insulin reduced the ketonaemia and the blood sugar level.

## DISCUSSION.

Sorbitol is antiketonic in liver slices of starved rats, but mannitol and dulcitol are not oxidized significantly and have no antiketogenic effect (Edson, 1936). The experiments reported here show that sorbitol is antiketogenic, whereas the other polyols are not, in anaesthetized rats made ketotic by various procedures.

Fructose is also antiketogenic in liver slices, but less effective than sorbitol. At the high blood concentrations attained by intravenous injection, fructose was just as effective as sorbitol *in vivo*.

The mechanism of the antiketogenic action of sorbitol is not clear, but it may be related to the reduction of DPN (see Blakley, 1952). It is now certain that DPN is concerned in the synthesis and oxidation of fatty acids (Lynen and Ochoa, 1953; Lynen, 1953). Possibly the phenomenon of ketosis is due to a decreased rate of fatty-acid synthesis, causing the diversion of acetyl-coenzyme A to acetoacetic-acid formation. Recent work has shown that impairment of glycolysis in liver, such as occurs in starvation (Lyon, Masri and Chaikoff, 1952) or diabetes (Baker, Chaikoff and Shusdek, 1952), diminishes the rate of fatty acid synthesis. Fructose, glycogen, hexose phosphates, and probably other substances whose metabolism is not blocked by a defect in the glucokinase reaction, can promote fatty-acid synthesis in liver preparations from alloxan diabetic rats (Shaw and Gurin, 1953). These facts suggest that normal glycolysis is needed to supply reduced DPN, presumably at the triosephosphate stage, for fatty-acid synthesis. The antiketogenic effect of sorbitol may therefore be due partly to the formation of fructose and partly to the formation of reduced DPN in the iditol-dehydrogenase reaction.

Small amounts of fructose appear in the blood of diabetic humans after the intravenous administration of sorbitol (Kilpatrick and Todd, 1953), supporting the view that the first step in sorbitol metabolism is conversion to fructose. Fructose is easily utilized by diabetic humans (Muller, Drucker, Owens, Craig and Woodward, 1952) and could be a useful source of readily available carbohydrate in the treatment of diabetic ketosis, but it is expensive and difficult to sterilize. Sorbitol, on the other hand, is non-toxic to humans (Ellis and Krantz, 1941), readily available and easily sterilized in solution. It can be administered intravenously in large amount (50–80 gm. in 10 p.c. solution) without ill effect (Smith, Finkelstein and Smith, 1940; Kilpatrick and Todd, 1953), and dissolved in the saline used to restore hydration in ketotic patients.

When it is administered slowly in 10 p.c. solution, the osmotic diuretic effect is slight.

From the above considerations it seems possible that intravenous sorbitol therapy may be useful as a safe adjunct to insulin therapy in the emergency treatment of severe diabetic ketosis.

## SUMMARY.

Rats have been made diabetic and ketotic by various procedures and the effects of sorbitol, mannitol, dulcitol, glycerol, fructose and glucose on the resulting ketonaemia have been examined after intravenous administration.

Sorbitol and fructose reduce the degree of ketonaemia in all cases, but mannitol and dulcitol are ineffective. Glycerol is effective but toxic.

The administration of sorbitol is suggested as a possible adjunct to insulin therapy in the treatment of human diabetic ketosis.

*Acknowledgment.* The author is most grateful to Prof. N. L. Edson for his interest, advice and criticism.

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# STUDIES ON *MYCOBACTERIUM ULCERANS* AND *MYCOBACTERIUM BALNEI*

## III. GROWTH IN THE SEMI-SYNTHETIC CULTURE MEDIA OF DUBOS AND DRUG SENSITIVITY *IN VITRO* AND *IN VIVO*.

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Antigenic studies of *Mycobacterium ulcerans* (Fenner, 1952; Fenner and Leach, 1952) suggested that this micro-organism differed serologically from other mycobacteria, but was more closely related to the mammalian tubercle bacilli than to other species examined. Cultural studies, reported in this paper, confirm this view.

Most of the investigations here described were carried out with the acid-fast bacterium recently described by Nordén and Linell (1951), as well as with *Mycobacterium ulcerans*. This micro-organism, tentatively named *Mycobacterium balnei* (Linell and Nordén, 1952), shares with *Myco. ulcerans* an inability to grow readily in culture media at 37° C., and the capacity to produce skin ulceration in man. The results reported here confirm the view of Nordén and Linell (1951) that the two species are distinct, as do studies of their behaviour in animals, to be described in a forthcoming paper.

### MATERIALS.

*Bacterial strains.*

*Myco. ulcerans* strains SF, RS, RT and KR (Fenner and Leach, 1952).

*Myco. tuberculosis* var. *humanis* strains H37Rv and H37Ra were kindly provided by Dr. R. J. Dubos.

*Myco. tuberculosis* var *bovis* strain BCG was kindly provided by the Commonwealth Serum Laboratories.

*Myco. phlei*—National Collection of Type Cultures, England, No. 525.

*Mycobacterium balnei*, of Nordén and Linell (1951), was kindly provided by Dr. A. Nordén.

Strain X: Isolated from the skin lesion of a patient.

Strain $K_2O$: Isolated from the cemented wall of a swimming-pool.

*Culture media.*

The semi-synthetic culture media of Dubos (Dubos and Middlebrook, 1947) were employed throughout.

*Mice.*

Multi-coloured mice, supplied by the University Breeding Establishment, were used when eight weeks of age.

## METHODS.

The methods used for the assessment of growth in the Tween-albumin medium have been described elsewhere in some detail (Fenner and Leach, 1953a). They are set out briefly below.

*Optical density* of the culture was determined with a Hilger "Spekker" photoelectric absorptiometer, using neutral glass filters, and standardizing against a blank consisting of uninoculated culture medium of the same batch as was used for the cultures. The optical density has been expressed in the Tables as the direct scale reading or a multiple thereof.

*Total bacterial counts* were made in a Petroff-Hausser chamber, using a reinforced coverslip and binocular phase contrast microscope with ocular ×20 and objective ×20.

*Dispersion of the bacilli* was determined by making differential counts of the numbers of bacilli per bacterial unit in films stained by the Ziehl-Neelsen method. The proportion of non-acidfast bacilli was determined at the same time.

*Measurement of the size of the bacilli* was carried out on a few preparations only. In these the centrifuged deposit from a culture was resuspended in petrol-ether, which produced satisfactory dispersion, and deposited on a slide. Examinations were made with phase-contrast illumination and an ocular micrometer.

## EXPERIMENTAL RESULTS.

### *Colonial morphology.*

Middlebrook, Dubos and Pierce (1947) systematized earlier observations upon the colonial morphology of the mammalian tubercle bacilli, and demonstrated the important correlation in these pathogens of virulence and the capacity to form serpentine cords. Examination of the growth of *Mycobacterium ulcerans* on the oleic-acid-albumin-agar solid medium and in liquid medium lacking Tween 80 showed that it formed cords identical in nature with those of virulent mammalian tubercle bacilli. Middlebrook and his collaborators found that the most sensitive cultural method of distinguishing between fully virulent mammalian tubercle bacilli, like H37Rv, and attenuated organisms, like BCG, was to incorporate 0·01 p.c. Tween 80 in the oleic-acid-albumin-agar medium. This had little if any effect on fully virulent strains, but colonies of the attenuated (and the avirulent) variants grown on such media were raised, smooth and opaque. Four strains of *Myco. ulcerans* were tested thus together with H37Rv, H37Ra, BCG and *Myco. balnei* with the results recorded in Table 1. All strains except *Myco. balnei* were grown on sections of the same plates, which were incubated at 33° C. for five weeks before examination. As *Myco. balnei* grew much more rapidly it was inoculated on separate plates which were examined after a week's incubation at 33° C.

In general, our results with the mammalian tubercle bacilli confirm those of Middlebrook, *et al.* (1947). All strains of *Myco. ulcerans* behaved in the same manner. Cording was, if anything, more distinct and persisted in the presence of higher concentrations of Tween 80 than with virulent human type H37Rv. In its colonial morphology on solid media *Myco. ulcerans* is indistinguishable

TABLE 1.

*Colonial morphology of* Myco. ulcerans, Myco. balnei *and virulent, attenuated, and avirulent strains of mammalian tubercle bacilli on oleic-acid-albumin-agar media containing various concentrations of Tween 80 and incubated at 33° C.*

| Strain | Period of incubation | | Concentration of Tween 80 | | | |
|---|---|---|---|---|---|---|
| | | | 0 p.c. | 0·05 p.c. | 0·01 p.c. | 0·02 p.c. |
| *Myco. tuberculosis* | | | | | | |
| H37Rv | | | spreading colony | raised centre | smoother | almost smooth |
| | 5 weeks | cording | +++ | ++ | + | – |
| BCG | | | central pimple | | | smooth, circular |
| | 5 weeks | cording | +++ | | | – |
| H37Ra | | | non-spreading, rough | slightly rough | smooth, circular | smooth, circular |
| | 5 weeks | cording | – | – | – | – |
| *Myco. ulcerans* | | | | | | |
| KR, SF, RS, RT | | | spreading colony | spreading | raised | almost smooth |
| | 5 weeks | cording | ++++ | +++ | ++ | ± |
| *Myco. balnei* | | | | | | |
| $K_2O$ "smooth" form | | | flat, glistening surface, central pimple, yellow pigmentation | smoother, more raised colony | smooth, raised | glistening surface, smooth, raised |
| | 7 days | cording | – | – | – | – |
| $K_2O$ "rough" form | | | dull surface, yellow pigmentation, central pimple | large central pimple | large central pimple, flatter outer portion | dull smooth surface, central pimple |
| | 7 days | cording | ++ | + | ± | – |

from the virulent mammalian tubercle bacilli. In liquid media lacking Tween 80 *Myco. ulcerans* also showed the pronounced cording characteristic of virulent mammalian tubercle bacilli.

The colonies of *Myco. balnei* were visible after three days' incubation at 33° C. and had attained a diameter of 3–5 mm., and a bright yellow pigmentation, by the seventh day. Pigmentation became evident more rapidly if the plates with well-grown colonies were left at room temperature for a couple of days instead of in the incubator.

Several colonial variants were recognized during serial passage of *Myco. balnei.* In the early stages of the investigation colonies on oleic-acid-albumin-agar had a glistening surface, a central raised area, and an outer thin portion which showed some indication of orientation of the bacilli, but nothing comparable to the "cording" characteristic of virulent mammalian tubercle bacilli. This is the "smooth" form referred to in Table 1. Later we observed a "rough" or "dry" variant of strain $K_2O$ and at about the same time Nordén (personal communication, 1953) observed a "dry" variant of a strain (B IV) isolated from the swimming pool. These variants produced colonies with a dull dry-looking surface, and something more nearly approaching the cording of virulent mammalian tubercle bacilli. Although there was little evidence of the serpentine arrangement of bacilli in well-grown colonies of *Myco. balnei,* this mode of growth was evident in flat young colonies if they were examined with high magnification (×100). Subsequent growth caused a "heaping-up" in the more central part of the colony and this obscured the serpentine arrangement. In liquid media lacking Tween 80 *Myco. balnei* grew in "cords" similar to those found with H37Rv grown in the same medium. In Tween-albumin liquid media the "dry" variant showed a much more rapid progress to growth in a pellicle than did the "smooth" strain. Both variants produced similar lesions in mice after both pad and intraperitoneal inoculation. A third variant was characterized by smooth, glistening and hemispherical colonies, with a narrow peripheral fringe.

The addition of Tween 80 to the solid medium had less effect on the roughness of the *Myco. balnei* colonies than on the serpentine arrangement of the tubercle bacilli.

*Cytochemical test.*

Dubos and Middlebrook (1948) showed that virulent mammalian tubercle bacilli could bind neutral red in alkaline suspensions, whereas avirulent variants, and non-pathogenic mycobacteria, lacked this capacity. The four strains of *Myco. ulcerans,* two strains of *Myco. balnei,* H37Rv, H37Ra, BCG and *Myco. phlei* were grown in lipid medium lacking Tween 80, to produce granular growth. The cytochemical test was carried out according to the technique described by Dubos and Middlebrook (1948), observations being made at 30 minutes and 24

hours. At the final reading the colour of the bacillary deposit was compared with the pH colour index of Hendon, Johnson & Sons Universal Indicator papers, and is recorded in Table 2 as 1, 2 or 3, equivalent to the colour indicating pH 1,

TABLE 2.

*Results of neutral red cytochemical test read 24 hours after addition of dye.*

| Bacterial strain | Reaction | Result Comparable Universal Indicator pH colour |
|---|---|---|
| *Myco tuberculosis* | | |
| H37Rv | Positive | 1–2 |
| BCG | Positive | 1–2 |
| H37Ra | Negative | 3 |
| *Myco. phlei* | Negative | 3 |
| *Myco. ulcerans* | | |
| KR | Positive | 1 |
| RT | Positive | 1 |
| SF | Positive | 1 |
| *Myco. balnei* | | |
| $K_2O$ | Negative | 3 |
| X | Negative | 3 |

2 or 3. The results shown in the Table indicate that whereas all strains of *Myco ulcerans* gave a strongly positive reaction, with a more intense red colouration than that of H37Rv, both strains of *Myco. balnei* were, like *Myco. phlei* and H37Ra, definitely negative. The two common colonial variants of *Myco. balnei* gave negative reactions.

*Growth of* Myco. ulcerans *and* Myco. balnei *in Tween-albumin-liquid medium at different temperatures of incubation.*

Strain SF of *Myco. ulcerans* and strain $K_2O$ of *Myco. balnei* were employed in these experiments.

Tween-albumin medium was dispensed in 5 ml. amounts in one-ounce screw-capped bottles ("universal containers"). The inocula were prepared by lightly centrifuging a two-weeks old culture of each strain (grown at 33° C.) to deposit the larger clumps. Using the supernatant suspension, aliquots of 0·5 ml. were inoculated into suitable numbers of bottles, which were then incubated at 33°, 35° and 37° C., and at room temperature (15°–16° C.). Heavy inocula were used so that growth could be followed photometrically throughout the experiments.

Two bottles were removed from each incubator daily, the contents pooled and total and viable counts, films and absorptiometer readings made.

The results of the experiments are summarized in Tables 3 and 4 and are shown graphically in Figs. 1 and 2.

TABLE 3.

*Growth of* Myco. ulcerans *(strain SF) in Tween-albumin liquid medium at different temperatures of incubation.*

| Age of culture (days) | 33° C | | | | 35° C. | | | | 37° C. | | | | Room temperature | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total* units | c.f.† | Total‡ bacilli | Opacity§ | Total units | c.f. | Total bacilli | Opacity | Total units | c.f. | Total bacilli | Opacity | Total units | c.f. | Total bacilli | Opacity |
| 0 | 69 | 2 | 138 | 31 | 69 | 2 | 138 | 31 | 69 | 2 | 138 | 31 | 69 | 2 | 138 | 31 |
| 1 | 62 | 2·5 | 156 | 36 | 82·5 | 2·6 | 214 | 36 | 60·5 | 2·5 | 151 | 36 | 76 | 2·5 | 190 | 34 |
| 2 | 71·5 | — | — | 44 | 87·5 | — | — | 37 | — | — | — | 34 | — | — | — | 29 |
| 3 | 119 | 3·6 | 428 | 66 | 72 | 3·5 | 252 | 54 | 63 | 2·4 | 151 | 33 | 70 | — | — | 34 |
| 4 | 90 | 3·7 | 333 | 88 | 62·5 | 3·7 | 231 | 42 | 57·5 | 3·9 | 224 | 32 | 78·5 | 2·2 | 173 | 40 |
| 5 | 119·5 | 2·9 | 347 | 128 | 86 | 2·4 | 206 | 36 | 78·5 | — | — | 34 | 64 | — | — | 31 |
| 6 | 221 | 9·3 | 2060 | 213 | 64 | 2·8 | 179 | 42 | 52·0 | 3·1 | 161 | 28 | 64 | — | — | 33 |
| 7 | 232 | 8·6 | 2750 | 280 | 69 | 2·6 | 179 | 43 | 67·5 | 3·1 | 209 | 34 | 60 | 2·8 | 168 | — |
| 14 | 1040 | 7·2 | 7490 | 980 | | | | | | | | | | | | |
| 21 | 1635 | 10·6 | 17330 | 1290 | | | | | | | | | | | | |
| 28 | 1536 | 3·6 | 5530 | 1180 | | | | | | | | | | | | |
| 33 | 5225 | 2·7 | 14100 | — | | | | | | | | | | | | |

* total count=bacterial units per ml. ×105.
† c.f.=clumping factor (average number of bacilli per bacterial unit).
‡ total bacilli=total count×clumping factor.
§ opacity=direct reading on absorptiometer ×1000.

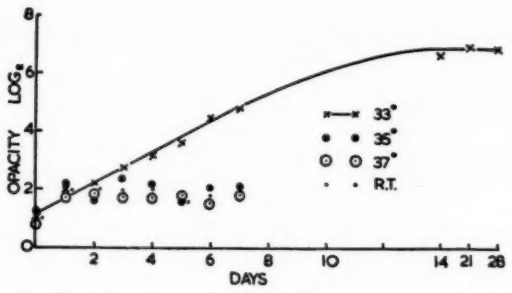


**Fig. 1.** Growth of *Myco. ulcerans* strain SF in Tween-albumin liquid medium at different temperatures of incubation. Bacterial density expressed as $\log_2$ (absorptiometer reading $\times 1000$). R.T.=room temperature (about 15° C.).

Myco. ulcerans.

When incubated at 33° C. *Myco. ulcerans* grew exponentially for about a week, and then entered phases of retardation and a stationary phase similar to those observed with mammalian tubercle bacilli growing in the same medium (Fenner and Leach, 1953a, b). Determination of the generation time from the graph (Fig. 1) showed that with *Myco. ulcerans* growing at 33° C., this was just under 48 hours. This may be compared with the very rough figure of 3–4 days for generation time *in vivo* calculated from the development of foot lesions in mice inoculated with different doses of bacilli (Fenner, unpublished) and the figures of 18 hours for H37Rv (Fenner and Leach, 1953a) and 20 hours for BCG (Fenner and Leach, 1953b) growing in the same Tween-albumin medium at 37° C. At temperatures other than 33° C. there was at no time an increase in the total count. The optical density rose slightly in some of the tubes incubated at 35° C., but there was no consistent upward trend even in this property.

In the cultures incubated at 33° C. there was an increase in the degree of clumping comparable with that observed with BCG (Fenner and Leach, 1953b), and a similar increased dispersion of the bacilli in the older cultures. No significant change occurred in the degree of clumping in the cultures incubated at other temperatures.

Measurement of the bacilli showed that, as with the tubercle bacilli (Fenner and Leach, 1953a, b), there was an initial phase of elongation, followed within a few days by a decrease in the size of the bacilli. No change in size occurred when the culture was incubated at 35° C. or 37° C. (Table 4).

Non-acidfast bacilli were not common at any stage. In the proliferating culture (33° C.) there were a few clumps (10 p.c. of the total) containing at least one non-acidfast bacillus in the 1, 2, 3 and 4-days old cultures, but after

TABLE 4.

*Changes in the length of* Myco. ulcerans *and* Myco. balnei *after incubation at different temperatures.*

| Strain | Temperature of incubation | Days after inoculation | Percentage of bacilli measuring 1·3 μ | 2·6 μ | 3·9 μ | 5·2 μ | 6·5 μ | 7·8 μ | Average length of bacilli (μ) |
|---|---|---|---|---|---|---|---|---|---|
| *Myco. ulcerans* | | | | | | | | | |
| SF | 33° C. | 0 | 2 | 44 | 40 | 14 | 0 | | 3·5 |
| | | 1 | 0 | 12 | 32 | 48 | 6 | 2 | 4·6 |
| | | 2 | 0 | 28 | 36 | 24 | 12 | | 4·2 |
| | | 3 | 0 | 30 | 38 | 22 | 8 | 2 | 4·1 |
| | | 4 | 0 | 38 | 34 | 22 | 6 | | 3·8 |
| | | 5 | 4 | 34 | 36 | 18 | 4 | 6 | 4·0 |
| | | 6 | 0 | 64 | 30 | 4 | 0 | 2 | 3·2 |
| | | 7 | 20 | 46 | 30 | 2 | 2 | | 2·9 |
| | 35° C. | 0 | 2 | 44 | 40 | 14 | 0 | | 3·5 |
| | | 1 | 2 | 42 | 38 | 18 | 0 | | 3·5 |
| | | 2 | 8 | 36 | 36 | 16 | 4 | | 3·5 |
| | | 3 | 14 | 44 | 30 | 12 | 0 | | 3·1 |
| | 37° C. | 0 | 2 | 44 | 40 | 14 | 0 | | 3·5 |
| | | 1 | 2 | 30 | 48 | 18 | 0 | | 3·6 |
| | | 2 | 6 | 36 | 36 | 22 | 0 | | 3·6 |
| *Myco. balnei* | | | | | | | | | |
| $K_3O$ | 33° C. | 0 | 22 | 58 | 20 | 0 | | | 2·6 |
| | | 1 | 0 | 28 | 50 | 20 | 2 | | 3·8 |
| | | 2 | 20 | 64 | 16 | 0 | | | 2·6 |
| | | 3 | 4 | 70 | 26 | 0 | | | 2·9 |
| | | 4 | 22 | 60 | 18 | 0 | | | 2·6 |
| | 35° C. | 0 | 22 | 58 | 20 | 0 | | | 2·6 |
| | | 1 | 0 | 20 | 40 | 26 | 14 | | 4·0 |
| | | 2 | 8 | 50 | 34 | 6 | 2 | | 3·2 |
| | | 3 | 20 | 68 | 12 | 2 | | | 2·6 |
| | *37° C. | 0 | 18 | 47 | 29 | 5 | 0 | | 2·9 |
| | | 1 | 5 | 51 | 31 | 10 | 2 | | 3·3 |
| | | 2 | 10 | 48 | 35 | 5 | 2 | | 3·1 |
| | | 3 | 10 | 47 | 32 | 11 | 0 | | 3·2 |
| | | 4 | 15 | 60 | 20 | 3 | 0 | | 2·8 |

* This experiment was carried out at a different time from the other experiments, using a different inoculum.

that none were seen, even in cultures incubated for five weeks. Non-acidfast bacilli were more common (in 10–20 p.c. of clumps) in the cultures incubated at 35° C. and 37° C.

Myco. balnei.

The temperature dependence of *Myco. balnei* was not as strict as that of *Myco. ulcerans*. There was undoubted growth, at about the same rate and to the same extent, at both 33° C. and 35° C. (Table 5, Fig. 2) and in the rack of bottles incubated at 37° C. the results varied considerably. Some bottles showed virtually no changes in the optical density or the total count even after several

TABLE 5.

*Growth of* Myco. balnei *(Strain $K_3O$) in Tween-albumin liquid medium at three different temperatures of incubation. Readings expressed as in Table 3.*

| Age of culture (days) | 33° C. | | | | 35° C. | | | | 37° C. | | | | Room temperature | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total units | c.f. | Total bacilli | Opacity | Total units | c.f. | Total bacilli | Opacity | Total units | c.f. | Total bacilli | Opacity | Total units | c.f. | Total bacilli | Opacity |
| 0 | 18·25 | 19 | 347 | 43 | 18·25 | 19 | 347 | 43 | 18·25 | 19 | 347 | 43 | 18·25 | 19 | 46·7 | 0043 |
| 1 | 21 | 10 | 210 | 210 | 39·5 | 11 | 435 | 200 | 30·5 | 5 | 153 | 177 | 19·5 | — | — | — |
| 2 | 42·5 | 11 | 468 | 600 | 54·5 | — | — | 310 | 60 | 8 | 480 | 620 | 20·5 | — | — | — |
| 3 | 166 | 3 | 498 | 590 | 204·1 | 5 | 1020 | 490 | — | — | — | 310 | — | — | — | — |
| 4 | 82·5 | 11 | 907·5 | 670 | 93 | 5 | 465 | 350 | 98<br>30·5 | 8 | 784<br>244 | 420<br>87 | — | — | — | — |
| 5 | 155 | — | — | 1180 | 261 | — | — | 750 | 200<br>39·5 | 8 | 1600<br>306 | 320<br>49 | 20 | — | — | — |
| 6 | 157 | 7 | 1097 | 1240 | 178 | 7 | 1240 | 540 | 62·5<br>39·5 | 8 | 500<br>306 | 160<br>36 | 20 | — | — | — |
| 7 | 168 | — | — | 1170 | 260 | 6 | 1560 | 920 | 45 | 8 | 360 | 54 | 37·5 | — | — | — |

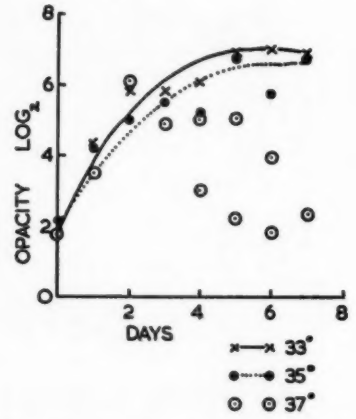


**Fig. 2.** Growth of *Myco. balnei* (strain K$_2$O) in Tweed-albumin liquid medium at different temperatures of incubation. Bacterial density expressed as log$_2$ (absorptiometer reading ×1000).

days' incubation, whereas in others the values were elevated, although not always as high as those of bottles incubated for the same length of time at 33° C.

In experiments made a year later than the foregoing, the bacterial strain being carried by serial passage at 33° C. in the intervening period, growth at 37° C. occurred in each of 25 bottles tested, though it was not as rapid as growth at 33° C. At 39° C. there was no growth in any of the 25 bottles tested.

As on the solid media, *Myco balnei* grew much faster in the Tween-albumin medium than did *Myco. ulcerans* or the tubercle bacilli. The data of the experiment just described are inadequate for an accurate determination of the generation time, as too few observations were made in the first two days, but it is apparent from Fig. 2 that at 33°, 35° C. and, in certain bottles, at 37° C. the generation time was about 11 hours. This agrees well with a value of 10–11 hours, worked out from the time of initial appearance of swelling of the foot in mice (Fenner, unpublished).

With the concentrations of Tween 80 used the degrees of dispersion of *Myco. balnei* varied considerably.

Films of *Myco. balnei* examined with the phase contrast microscope showed that the bacilli were elongated at the end of the first day, and thereafter diminished in length (Table 4). In contrast to the results observed with *Myco. ulcerans,* this occurred in cultures incubated at 35° as well as at 33° C. At 37° C. there was a slight shift to the right at the end of the first day (i.e., a diminution in the number of very short bacilli and a slight increase in the average length of the bacilli). Although this was confirmed on repeated counts, it was a very slight change compared with that observed at 33° C. and 35° C.

Non-acidfast bacilli were not common in cultures incubated at 33° and 35° C., occurring in 10–20 p.c. of clumps throughout the growth period. They were somewhat more common in the culture incubated at 37° C., occurring in about 50 p.c. of clumps.

*Drug-sensitivity of* Myco. ulcerans *and* Myco. balnei.

Both *Myco. ulcerans* and *Myco. balnei* may cause troublesome skin ulceration in man (MacCallum, *et al.*, 1948; Nordén and Linell, 1951). Determination of their reaction to anti-tuberculous drugs was justified, therefore, to provide a basis for rational chemotherapy. In addition, it has been suggested (Cattaneo, Morellini, Ortali and Penso, 1950; Lutz, 1950; Buraczewska, Kurylowicz and Woznicka, 1952) that saprophytic mycobacteria might be differentiated from virulent tubercle bacilli by their differing resistance to drugs.

The plan of the experiments was similar throughout. The required concentrations of each of the four drugs tested, streptomycin, para-amino-salicylic acid (PAS), para-acetamidobenzaldehyde (thiosemicarbazone), and isonicotinic acid hydrazide (isoniazid), were incorporated in plates of oleic-acid-albumin-agar medium to the required final concentrations. Streptomycin was incorporated by dissolving the dry powder in sterile distilled water, PAS by converting to

TABLE 6.

*The sensitivity of* Myco. ulcerans *and* Myco. balnei *to streptomycin.*

| Strain | Concentration of inoculum | Concentration of streptomycin (μg. per ml.) 10 | 1 | 0·1 | 0·05 | 0 |
|---|---|---|---|---|---|---|
| *Myco. tuberculosis* | | | | | | |
| H37Rv | concentrated | 0 | 28 (D.G.)* | +++* | +++ | +++ |
| | dilute | 0 | 0 | 30 | 20 | 25 |
| *Myco. ulcerans* | | | | | | |
| RS | concentrated | 0 | 0 | +++ (D.G.) | +++ | +++ |
| | dilute | 0 | 0 | 9 (D.G.) | 8 | 7 |
| RT | concentrated | 0 | 0 | +++ (D.G.) | +++ | +++ |
| | dilute | 0 | 0 | 35 (D.G.) | 35 (D.G.) | 50 |
| KR | concentrated | 0 | 0 | +++ (D.G.) | +++ | +++ |
| | dilute | 0 | 0 | 70 (D.G.) | 70 | 60 |
| SF | concentrated | 0 | 0 | 25 (D.G.) | +++ | +++ |
| | dilute | 0 | 0 | — | 15 | 30 |
| *Myco. balnei* | | | | | | |
| $K_2O$ | concentrated | 0 | +++ | +++ | +++ | +++ |
| | dilute | 0 | 12 (D.G.) | 14 | 15 | 17 |
| X | concentrated | 0 | +++ | +++ | +++ | +++ |
| | dilute | 0 | 14 (D.G.) | 16 | 12 | 17 |

*28, etc.=average colony count.
+++=semiconfluent to confluent growth.
D.G.=delayed growth.
0=no growth.
—=test unsatisfactory

TABLE 7.

*The sensitivity of* Myco. ulcerans *and* Myco. balnei *to para-amino-salicylic acid (P.A.S.).*

| Strain | Concentration of inoculum | Concentration of PAS (μg. per ml.) 100 | 25 | 10 | 1 | 0·025 | 0·0125 | 0 |
|---|---|---|---|---|---|---|---|---|
| *Myco tuberculosis* | | | | | | | | |
| H37Rv | concentrated | 0 | 0 | 0 | — | +++ (D.G.) | +++ | +++ |
| | dilute | 0 | 0 | 0 | 20 (D.G.) | 20 (D.G.) | — | 25 |
| *Myco ulcerans* | | | | | | | | |
| RS | concentrated | +++ (D.G.) | +++ (D.G.) | +++ (D.G.) | +++ (D.G.) | +++ | +++ | +++ |
| | dilute | 0 | 0 | 8 (D.G.) | 9 (D.G.) | 12 | — | 7 |
| RT | concentrated | — | — | +++ (D.G.) | +++ | +++ | +++ | +++ |
| | dilute | — | — | 50 (D.G.) | 50 (D.G.) | 60 | 60 | 50 |
| KR | concentrated | — | — | 0 | — | +++ | +++ | +++ |
| | dilute | — | — | 0 | 20 (D.G.) | 60 | 70 | 60 |
| SF | concentrated | — | — | +++ (D.G.) | — | +++ | +++ | +++ |
| | dilute | — | — | 40 (D.G.) | 40 | 20 | — | 30 |
| *Myco balnei* | | | | | | | | |
| $K_2O$ | concentrated | +++ | +++ | +++ | +++ | — | — | +++ |
| | dilute | 17 | — | 18 | 18 | — | — | 16 |
| X | concentrated | +++ | +++ | +++ | +++ | — | — | +++ |
| | dilute | 12 | — | 8 | 14 | — | — | 17 |

Symbols as in Table 6.

TABLE 8.

*The sensitivity of* Myco. ulcerans *and* Myco. balnei *to thiosemicarbazone.*

| Strain | Concentration of inoculum | Concentration of thiosemicarbazone (μg. per ml.) 20 | 10 | 1 | 0·1 | 0·05 | 0 |
|---|---|---|---|---|---|---|---|
| *Myco. tuberculosis* | | | | | | | |
| H37Rv | concentrated | — | 0 | 32 (D.G.) | +++ (D.G.) | +++ | +++ |
| | dilute | — | 0 | 0 | 9 | 16 | 25 |
| *Myco. ulcerans* | | | | | | | |
| RS | concentrated | +++ | — | +++ | +++ | +++ | +++ |
| | dilute | 6 | 10 | 12 | 11 | 6 | 7 |
| RT | concentrated | — | +++ | +++ | +++ | +++ | +++ |
| | dilute | — | — | 45 | 50 | 60 | 50 |
| KR | concentrated | +++ | +++ | +++ | +++ | +++ | +++ |
| | dilute | — | 70 (D.G.) | 60 | 55 | 60 | 60 |
| SF | concentrated | — | — | +++ | +++ | +++ | +++ |
| | dilute | — | 9 | 25 | 35 | 40 | 40 |
| *Myco. balnei* | | | | | | | |
| $K_2O$ | concentrated | +++ | — | +++ | — | — | +++ |
| | dilute | 17 | 16 | 15 | — | — | 16 |
| X | concentrated | +++ | — | +++ | — | — | +++ |
| | dilute | 10 | 16 | 11 | — | — | 17 |

Symbols as in Table 6.

TABLE 9.

*The sensitivity of* Myco. ulcerans *and* Myco. balnei *to isonicotinic acid hydrazide.*

| Strain | Concentration of inoculum | Concentration of isonicotinic acid hydrazide (μg. per ml.) 10 | 1 | 0·1 | 0·05 | 0·0125 | 0 |
|---|---|---|---|---|---|---|---|
| *Myco. tuberculosis* | | | | | | | |
| H37Rv | concentrated | 0 | 0 | 0 | 2 | +++ | +++ |
| | dilute | 0 | 0 | 0 | 0 | +++ | +++ |
| *Myco. ulcerans* | | | | | | | |
| RS | concentrated | +++ (D.G.) | +++ | +++ | — | +++ | +++ |
| | dilute | — | 30 | 35 | 37 | 42 | 34 |
| RT | concentrated | +++ (D.G.) | +++ | +++ | +++ | +++ | +++ |
| | dilute | — | 20 | 24 | 25 | 22 | 28 |
| KR | concentrated | +++ (D.G.) | +++ | +++ | +++ | +++ | +++ |
| | dilute | — | 12 | 12 | 10 | 8 | 11 |
| SF | concentrated | +++ (D.G.) | +++ | +++ | +++ | +++ | +++ |
| | dilute | — | 28 | 16 | 17 | 25 | 18 |
| *Myco. balnei* | | | | | | | |
| $K_2O$ | concentrated | +++ (D.G.) | +++ | +++ | +++ | — | +++ |
| | dilute | 5 | 30 | — | 35 | — | 30 |
| X | concentrated | +++ (D.G.) | +++ | +++ | +++ | — | +++ |
| | dilute | 11 | 27 | — | 38 | — | 35 |

Symbols as in Table 6.

the soluble sodium salt with N/1 NaOH, neutralizing with dilute HCl and sterilizing by Seitz filtration, thiosemicarbazone by dissolving with heating in propylene glycol and diluting 4/1 with sterile distilled water, and isoniazid by dissolving in water and sterilizing by boiling.

Each strain tested, viz., *Myco. tuberculosis* strain H37Rv, *Myco. ulcerans* strains SF, RS, KR, RT and *Myco. balnei* strains X and $K_2O$, were inoculated on sectors of the same plates, except that separate plates were used for the rapidly growing *Myco. balnei*. Two dilutions of each culture were used, one which was expected to give confluent growth in the control drop and the other countable numbers of colonies, and one plate was used for each dilution. All plates were incubated at 33° C. Counts were made after five days in the case of *Myco. balnei* and 4–6 weeks for *Myco. ulcerans* strains. All plates showing no growth at the first examination were re-examined with the colony microscope three weeks later. The first three drugs were tested simultaneously with the same preparations of bacilli, but the experiment with isoniazid was performed later, with another set of cultures.

The results are shown in Tables 6–9. The results with H37Rv, the control organism, showed that the plates contained the indicated concentrations of the different drugs, which were active. *Myco. ulcerans* was as sensitive to streptomycin as H37Rv, being inhibited by 1 μg. per ml. *Myco. balnei* was unaffected by 1 μg. per ml., but completely suppressed by 10 μg. per ml. H37Rv was completely suppressed by 10 μg. PAS per ml., and growth was delayed with much smaller amounts. *Myco. balnei* was hardly affected by as much as 100 μg. per ml., confirming the results of Lehmann (Nordén and Linell, 1951), and *Myco. ulcerans* showed delayed growth with 10 μg. per ml., and inhibition of small inocula by 25 μg. per ml., but was not as sensitive as H37Rv. There were clear-cut differences with both thiosemicarbazone and isoniazid. H37Rv was partially inhibited by small doses of each (1·0 μg. per ml. of thiosemicarbazone, 0·05 μg. per ml. isoniazid), whereas both *Myco. ulcerans* and *Myco. balnei* appeared to be unaffected by those drugs, except that with the highest concentration of isoniazid (10 μg. per ml.) there was delayed growth.

The activity of streptomycin *in vitro* was confirmed by *in vivo* tests. Mice were inoculated in one foot pad with concentrated suspensions of *Myco. ulcerans* strain KR and *Myco. balnei* strain $K_2O$. Three different treatment schedules were followed, mice being inoculated intraperitoneally with 1·5 mg. of streptomycin daily for six days each week. In one group treatment commenced on the same day as the mice were inoculated, in another treatment was begun as soon as swelling of the inoculated foot was visible, and in the third group treatment was withheld until the foot lesion had reached an advanced stage.

With both *Myco. ulcerans* and *Myco. balnei* mice treated from the day of infection, or when early foot lesions only were present, showed at the most very slight symptoms of infection. Treatment of the advanced foot lesions had

no effect in *Myco. ulcerans* infections, although this experiment was terminated by intercurrent infection three weeks after treatment had begun. The pustular foot lesions, due to *Myco. balnei*, regressed more rapidly in the treated animals than in the controls, but these also subsequently regressed.

## DISCUSSION.

The results here reported confirm Nordén and Linell's (1951) belief that *Myco. ulcerans* and *Myco. balnei* are not identical. There were close resemblances between *Myco. ulcerans* and virulent mammalian tubercle bacilli, particularly the colonial morphology on solid medium and the strong positive reaction to the cytochemical tests.

The generation time of *Myco. ulcerans* is longer than that of the mammalian tubercle bacilli, and this is not a reflection merely of the lower temperature of incubation, for at 33° C. H37Rv was consistently observed to multiply much more rapidly than *Myco. ulcerans* (Fenner, unpublished). There appears to be no definite evidence of growth of *Myco. ulcerans* at 35° C. and 37° C., or at room temperature. There was no consistent change in the optical density and microscopic examination of the bacilli showed that they did not elongate during the first few hours of incubation.

*Myco. balnei* can be easily differentiated from *Myco. ulcerans*. In all its properties (including its occurrence in slime) it resembles a saprophytic mycobacterium which possesses the ability to proliferate in suitable tissues of animals. The temperature range for growth is fairly wide, although growth occurs more regularly and more rapidly at 33° C. than at 37° C.; and the generation time is short, about 11 hours. The colonial morphology differed from that of the virulent mammalian tubercle bacilli, and the colonies became deeply pigmented after about seven days. The neutral red reaction was negative.

Several European investigators (Penso, 1950; Lutz, 1950; Buraczewska *et al.*, 1952) have commented upon the insensitivity of saprophytic mycobacteria ("paratubercle bacilli") to the synthetic drugs (PAS, thiosemicarbazone) compared with their sensitivity to streptomycin. Penso, indeed, suggested that sensitivity to PAS should be used as a criterion to separate pathogenic tubercle bacilli from the other mycobacteria, although later he found (Penso, Castelnuovo, Gaudiano, Princivalle, Vella and Zampieri, 1952) that a new mycobacterium, which was named *Myco. minetti*, was pathogenic in several laboratory animals although it was quite resistant to PAS.

*Myco. balnei* was virtually unaffected by PAS, thiosemicarbazone and isoniazid, but was susceptible to streptomycin, and thus resembled the "paratubercle bacilli" of the above authors. *Myco ulcerans* was also insensitive to thiosemicarbazone and isoniazid, but gave irregular results with PAS, one strain being completely inhibited by 10 $\mu$g. per ml. and the other three partly inhibited at this

concentration. The only strain tested at higher concentrations showed greatly retarded growth with concentrated inocula and complete inhibition with small inocula. *Myco. ulcerans* was very sensitive to streptomycin.

*In vivo* experiments, utilizing footpad inoculations of mice, showed that infection with mycobacteria could be controlled by streptomycin if the lesions had not progressed too far before treatment was commenced.

## SUMMARY.

The cultural behaviour of *Myco. ulcerans* and *Myco. balnei* has been studied on the semisynthetic media of Dubos. *Myco. ulcerans* showed well developed cording of the type described for virulent mammalian tubercle bacilli grown on this medium, whereas well-grown colonies of *Myco. balnei* showed no such arrangement of the bacilli, and were bright yellow in colour. The positive reaction of *Myco. ulcerans* and the negative reaction of *Myco. balnei* to the neutral red test of Dubos accord with these different modes of growth.

In liquid media *Myco. ulcerans* grew exponentially at 33° C., its generation time being about 48 hours. At higher temperatures (35° and 37° C.), and at room temperature (about 15° C.), no growth occurred. Growth of *Myco balnei* was less strictly controlled by the temperature of incubation and good growth occurred at 33°, 35° and, irregularly, at 37° C. The generation time at 33° C. and 35° C. was about 11 hours.

Both species differed from *Myco. tuberculosis* var. *humanis* strain H37Rv in their *in vitro* sensitivity to drugs. *Myco ulcerans* was highly sensitive to streptomycin and relatively sensitive to PAS, but insensitive to thiosemicarbazone and isonicotinic acid hydrazide, whereas *Myco. balnei* was slightly less sensitive than the other species to streptomycin, and insensitive to PAS, thiosemicarbazone and isonicotinic acid hydrazide.

*In vivo* tests, utilizing footpad inoculation of mice, indicated that infection with both *Myco. ulcerans* and *Myco. balnei* could be controlled with streptomycin as long as the lesions had not developed too greatly before treatment was commenced.

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# THROMBIN FORMATION AND YIELD IN SHED BLOOD IN RELATION TO THROMBOPLASTINS AND PROTHROMBOPLASTINS[1]

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The experiments to be described were undertaken to provide an understanding of the defect in haemorrhagic tendencies connected with the thromboplastin complex. In severe haemorrhagic conditions, due to a deficiency of a clotting component, laboratory findings usually correlate well with clinical symptoms, but in a few cases it is difficult to decide whether the haemorrhages are due to a deficiency of a clotting component or to other causes. This is particularly noticeable in suspected thromboplastin deficiencies, because of the lack of techniques which give quantitative results. Blood contains at least two plasma precursors of thromboplastin; anti-haemophilic globulin (Lewis *et al.*, 1946; Brinkhous *et al.*, 1951) and plasma thromboplastin component (PTC), also known as Christmas factor (Aggeler *et al.*, 1952; Biggs *et al.*, 1952). The two components have been called $\alpha$- and $\beta$-prothromboplastin respectively (Fantl and Sawers, 1954). These are converted on certain surfaces to $\alpha$- and $\beta$-thromboplastin; the latter two, as well as adequate numbers of thrombocytes and calcium ions, form the blood thromboplastin complex. This reacts with the prothrombin system and thrombin is formed. In addition, blood contains factors which inactivate thrombin. The amount of thrombin which is present in clotting blood at a given moment depends therefore on the balance between formation and inactivation. Under these conditions it is obviously not possible to obtain quantitative results. Recently it was shown that phenols have the property of delaying the inactivation of thrombin by plasma (Fantl, 1954). In the presence of phenols it should be possible to assess quantitatively the ability of a system to form thrombin. With this device the relationship between thromboplastin activity and rate of formation and yield of thrombin was investigated.

## EXPERIMENTAL.

Human venous blood was obtained by puncture of an arm vein. Arterial blood was aspirated from the femoral artery. In both cases the first few ml. were discarded. Nine

[1] Part of the expenses of this investigation were defrayed by a grant from the National Health and Medical Research Council, Australia.

volumes of blood were mixed immediately with one volume of 0·1 M-sodium oxalate or with one volume of 0·13 M-trisodium citrate. Capillary blood was obtained from a finger or the heel, using a spring lancet which produced a 4 mm. deep puncture. 0·18 ml. of blood were drawn into a pipette which contained 0·02 ml. of 0·1 M-sodium oxalate. The contents were delivered into 0·8 ml., 0·15 M-sodium chloride.

Oxalated plasma was mixed with 10 p.c. of its volume of freshly prepared barium sulphate suspension which removes $\beta$-prothromboplastin (PTC, Christmas factor). After stirring in an ice-bath for 30 minutes, clear plasma was obtained by centrifugation at +6° C. This plasma will be called Ba-plasma, and was used both as a source of $\alpha$-thromboplastin (anti-haemophilic factor) and fibrinogen.

"Stored plasma" was obtained from oxalated plasma, to which was added 0·01 p.c. merthiolate. Approximately 20 ml. were kept in the dark in test tubes plugged with cotton wool at +20° C. As plasma stored for at least 10 days is free of $\alpha$-prothromboplastin, this was used as a source of $\beta$-thromboplastin.

Thrombocytes, isolated by differential centrifugation from citrated human blood, were washed free of protein with a solution containing 0·024 M-trisodium citrate and 0·085 M-sodium chloride. Finally a suspension in 0·15 M-sodium chloride was prepared. To provide a source of tissue thromboplastin, pieces of abdominal aorta and portal vein were removed from a rabbit under nembutal anaesthesia. The isolated blood vessels were washed free of blood with 0·15 M-sodium chloride, cut into small pieces and ground into a suspension.

Reagents and details for the determination of prothrombin, and the relationship between thrombin clotting time and thrombin units are given by Fantl (1954).

### *Intrinsic ability of clotting blood to form thrombin.*

Experiments were designed to determine the maximum amount of thrombin which can be produced by blood. In Fig. 1 are given results of experiments concerned with the rate of formation and yield of thrombin in diluted blood in the presence of 0·03 M-pyrocatechol.

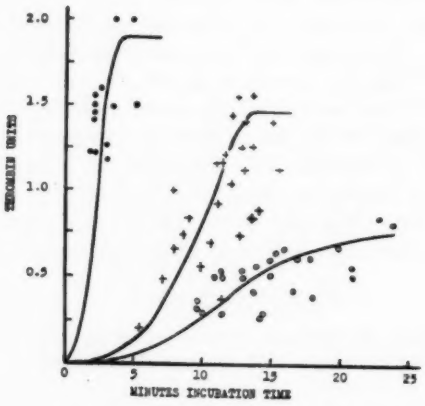



Fig. 1

Composition of reaction mixtures: (1) 0·06 ml. human venous oxalated blood, 0·69 ml., 0·15 M-sodium chloride, 0·15 ml. 6 p.c. human brain suspension in 0·15 M-sodium chloride, 0·6 ml. 0·025 M-calcium chloride, 0·3 ml. 0·18 M-pyrocatechol in veronal buffer pH 7·4 (Curve ●——●).

(2) Reaction mixture as in (1) except that brain suspension was omitted and replaced by 0·15 ml. 0·15 M-sodium chloride (Curve +——+).

(3) Reaction mixture as in (2) except that pyrocatechol was replaced by 0·3 ml. veronal buffer pH 7·4 (Curve O——O).

In each of the three cases, at known periods, 0·2 ml. were removed, blown into a mixture of 0·2 ml. human Ba-plasma and 0·05 ml. 0·18 M-pyrocatechol in veronal buffer pH 7·4, and the clotting time determined. The reactions were carried out in a water bath at 28° C. ±1°. The clotting times were converted into thrombin units.

The curves given in Fig. 1 represent average results obtained from 5 venous blood specimens taken from normal human donors. It will be noted that in the presence of brain thromboplastin and pyrocatechol (curve ●——●) maximum thrombin formation occurred in 2–5 minutes incubation time. The yield varied between 1·25 and 2 thrombin units per 0·2 ml. reaction mixture corresponding to 0·0066 ml. oxalated blood. In the absence of brain extract and presence of pyrocatechol (curve +——+) maximum thrombin formation occurred in 12–14 minutes, and average yield of thrombin was 86 p.c. (varying from 80–100 p.c.) of that in the former instance. In the absence of both brain thromboplastin and pyrocatechol (curve O——O) maximum thrombin formation occurred in 18–24 minutes, and the yield was 42 p.c. of that obtained in the first instance. The smaller rate of thrombin formation, as well as the lower yield in the last case, are due to antithrombin activity. From these observations it appears that the blood thromboplastin complex is as effective as brain extract in converting the prothrombin present into thrombin. The latter can be measured quantitatively in the presence of pyrocatechol.

It has been postulated (Fantl and Hayes, 1953) that circulating blood contains the precursors of thromboplastin, which on certain surfaces become activated. This activation should result in a faster rate of thrombin formation. This assumption was tested with oxalated blood, which was kept in pyrex glass tubes at room temperature for varying periods. The results of such experiments are given in Fig. 2.

One volume of human oxalated venous blood was immediately after collection diluted with four volumes 0·15 M-sodium chloride and kept at +20° C. 0·2 ml. were added to a reaction mixture similar in composition to that in (2), Fig. 1. Rate of thrombin formation 10 minutes after blood collection (curve +——+). The same 60 minutes after collection (curve ●——●). The same 120 minutes after collection (curve O——O).

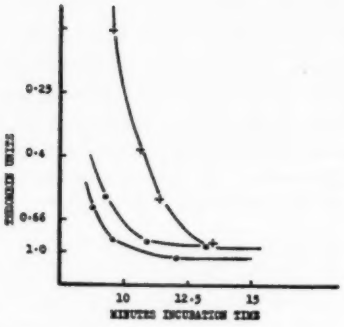



Fig. 2

It will be seen from Fig. 2 that diluted oxalated blood when recalcified 10 minutes after collection produced its maximum yield of thrombin in approximately 13 minutes, whereas after 60 and 120 minutes after collection the time was shortened to 11 minutes.

These experiments indicate that in order to obtain comparable results it is necessary to determine the rate of thrombin formation immediately after the collection of blood. In another experiment thrombocyte counts were carried out simultaneously with the rate of thrombin formation. No change in their numbers was observed. These facts are taken as further support for the assumption that activation of prothromboplastin to thromboplastin occurs on glass surfaces without breakdown of thrombocytes.

At times it may be desirable to examine capillary blood. The rates of thrombin formation of capillary blood compared with that of venous blood are given in Fig. 3. 0·04 ml. human oxalated venous blood and 0·71 ml. 0·15

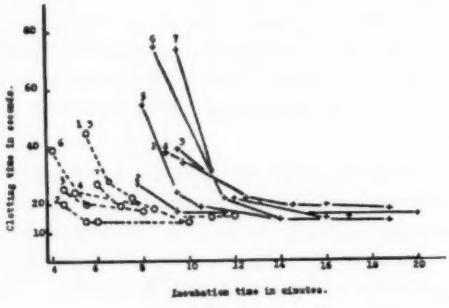


Fig. 3

M-sodium chloride, otherwise similar reaction mixture as used in (2) Fig. 1 (curve +——+). 0·04 ml. oxalated capillary blood, otherwise the same reaction mixture as before (curves O - - - O).

The curves in Fig. 3 represent results obtained from seven pairs of venous and capillary blood. It is apparent that the rate of thrombin formation is faster in capillary blood than in venous blood, but the yield of thrombin, as judged by the final clotting time, is identical in both cases. In order to find the cause of the faster rate of thrombin formation in capillary blood when compared with venous blood, the process was studied in venous, arterial and capillary blood taken from the same donor. Results are given in Fig. 4.

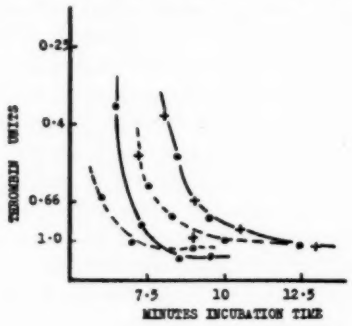



Fig. 4

Experimental conditions similar to those given in Fig. 3. Venous blood (curves ●···●), arterial blood (curves +—+), capillary blood (curves o—o). The two curves with full lines were obtained 45 minutes after blood collection. The curves with dotted lines were obtained 105 minutes after blood collection.

In other experiments suspensions of isolated arteries and veins obtained from a rabbit were added to diluted blood. The results of these experiments and a comparison with brain thromboplastin is made in Fig. 5.

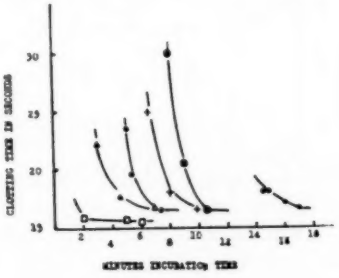



Fig. 5

0·04 ml. human venous oxalated blood was used. The composition of the reaction mixture was similar to that given in (2) Fig. 1. Venous blood 6 minutes after collection (curve ●——●). Venous blood 50 minutes after collection (curve ⊙——⊙). Venous blood plus 1 ml. rabbit's aorta suspension 4 minutes after blood collection (curve +——+). Venous blood plus 1 ml. rabbit's vein suspension 2 minutes after blood collection (curve O——O). Venous blood plus 0·03 p.c. human brain suspension 54 minutes after blood collection (curve △——△). Venous blood plus 3 p.c. human brain suspension 34 minutes after blood collection (curve □——□).

The results given in Fig. 4 indicate that the rate of thrombin formation is the same in venous and arterial blood. Fig. 5 shows that damaged blood vessels contribute thromboplastin. The greater rate of thrombin formation in capillary blood than in systemic blood is therefore probably due to contamination of capillary blood with tissue thromboplastin. A quantitative comparison of the activities of the various thromboplastin preparations shows that brain extract was the fastest reacting thromboplastin (curves △——△ and □——□). Somewhat slower reacting were the blood vessel suspensions (curves +——+ and O——O). The blood thromboplastin complex showed the slowest rate of thrombin formation. The yield of thrombin was of the same magnitude in all these experiments. Again it will be seen from the results given in Figs. 4 and 5 that the rate of thrombin determined in diluted blood 6 minutes after collection is slower than 50 minutes later.

In further work the contribution of each of the several components of the thromboplastin complex of blood was investigated with regard to their influence on the thrombin formation rate of shed blood.

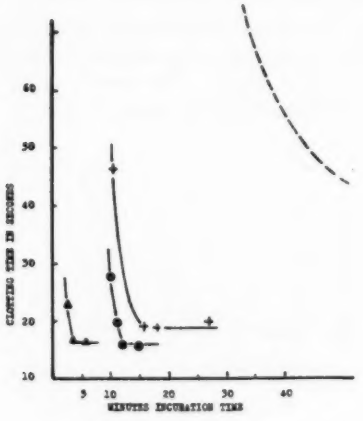



Fig. 6

*The effect of thrombocytes on the rate of formation and yield of thrombin.*

In the following experiment freshly isolated thrombocytes were used. Another component of the reaction mixture was freshly prepared Ba-plasma which contributed $\alpha$-prothromboplastin and prothrombin accelerator. Finally, stored plasma was added as a source of $\beta$-prothromboplastin, prothrombin and the various activators, termed SPCA by Alexander *et al.* (1948), co-thromboplastin by Mann and Hurn (1951), Factor 7 by Koller *et al.* (1951), and proconvertin by Owren (1951). Results are plotted in Fig. 6.

Composition of reaction mixtures: (1) 0·02 ml. Ba-plasma, 0·02 ml. plasma stored for seven days, 0·15 ml. 0·06 p.c. human brain extract. Other components similar to those in (1) Fig. 1 (curve ▲——▲). (2) The same as (1) except that brain extract was replaced by 0·1 ml. of a suspension containing $3\times10^5$ mm.$^3$ freshly prepared thrombocytes (curve ●——●). (3) The same as (1) except that $6\times10^4$ mm.$^3$ thrombocytes were added (curve +——+). (4) 0·02 ml. Ba-plasma, 0·02 ml. stored plasma without any thromboplastin addition, otherwise similar to (1) (curve – – – –). Total reaction mixture in each case 1·8 ml. Thrombin determination was carried out in 0·2 ml. reaction mixture.

The results given in Fig. 6 indicate that 0·2 ml. of a reaction mixture containing Ba-plasma and stored plasma in the absence of thrombocytes, produced 0·24 units of thrombin in 50 minutes incubation time (curve – – – –). This represents 27 p.c. of 0·92 units, which were obtained with brain extract in 3 minutes (curve ▲——▲). In the presence of $1\cdot7\times10^4$ thrombocytes per mm.$^3$ of reaction mixture, 0·88 units of thrombin were formed in 11 minutes incubation time (curve ●——●) corresponding to a yield of 96 p.c., which fell to 0·7 units, equal to 80 p.c. after 15 minutes' incubation time in the presence of $0\cdot34\times10^4$ thrombocytes (curve +——+).

Having observed a marked influence of fresh thrombocytes on the rate of formation as well as on the yield of thrombin, it was of interest to see how

TABLE 1.

*Influence of various thrombocyte preparations on the rate of formation and yield of thrombin.*

| | Thromboplastin α- | Thromboplastin β- | Thrombocyte preparations | Thrombin formation Mins. | Thrombin formation Units per ml. | Thrombin formation Yield p.c. |
|---|---|---|---|---|---|---|
| 1. | 0·1 | 0·1 | 0·1 fresh $3\times10^5$ mm.$^3$ | 11 | 396 | 99 |
| | 0·0 | 0·1 | 0·1 fresh $3\times10^5$ mm.$^3$ | 35 | 297 | 75 |
| | 0·1 | 0·1 | Nil | 50 | 108 | 27 |
| 2. | 0·1 | 0·1 | 0·1 fresh $2\times10^5$ mm.$^3$ | 20 | 250 | 65 |
| | 0·0 | 0·1 | 0·1 fresh $2\times10^5$ mm.$^3$ | 37 | 100 | 40 |
| 3. | 0·1 | 0·1 | 0·1 one day 6° C. $3\times10^5$ mm.$^3$ | 23 | 280 | 82 |
| | 0·1 | 0·1 | 0·02 one day 6° C. $3\times10^5$ mm.$^3$ | 25 | 216 | 64 |
| 4. | 0·1 | 0·1 | 0·1 one day −15° C. $3\times10^5$ mm.$^3$ | 18 | 315 | 88 |
| | 0·0 | 0·1 | 0·1 one day −15° C. $3\times10^5$ mm.$^3$ | 50 | 257 | 70 |
| | Whole blood | | | | | |
| 5. | 0·04 ml. | | $10\times10^3$ mm.$^3$ | 50 | 96 | 60 |
| | 0·04 ml. | | 10+0·1 ml. fresh $4\times10^5$ mm.$^3$ | 16 | 160 | 100 |

certain physical treatment would influence their activity. Results obtained with thrombocytes stored at +6° C., or frozen at −15° C. and thawed, are compared in their activity with thrombocytopenic blood and with fresh preparations.

The composition of the reaction mixtures was similar to that given in Fig. 6. The source of β-prothromboplastin in No. 1 experiment was a plasma stored for 1 week, in No. 2 it was stored for 4 weeks, in No. 3 for 3 weeks, and in No. 4 for 2 weeks.

The results given in Table 1 indicate that the maximum yield of thrombin was only obtained when the three components Ba-plasma, stored plasma and thrombocytes were present in adequate concentration. A reduction of any one of them lowered the rate of formation and yield of thrombin, which is calculated on the assumption that brain thromboplastin yielded 100 p.c. thrombin. In Experiment 5 blood from a thrombocytopenic patient was taken. It is apparent that despite the severe reduction of thrombocytes in this patient's blood, 60 p.c. of prothrombin was converted into thrombin, although the rate of formation of thrombin was considerably slower than normal. Addition of adequate numbers of thrombocytes produced a normal rate of formation and yield of thrombin.

In another experiment the effect of fresh and heated thrombocytes was compared. A portion of a suspension of freshly prepared human thrombocytes containing $37\times10^4$ thrombocytes per mm.$^3$ was heated at 65° C. for 20 minutes. Oxalated plasma, poor in thrombocytes, was prepared from the blood of a case of complete α-prothromboplastin deficiency (haemophilia). This was used as a source of β-prothromboplastin, and freshly prepared Ba-plasma was the source of α-prothromboplastin. The results are given in Fig. 7.

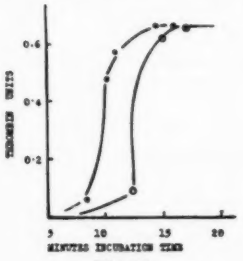



Fig. 7

Composition of reaction mixture: 0·02 ml. patients' plasma, 0·01 ml. Ba-plasma, 0·1 ml. freshly prepared thrombocytes containing $37\times10^4$ mm.$^3$ (curve ●——●). 0·02 ml. patients' plasma, 0·01 ml. Ba-plasma, 0·1 ml. thrombocytes heated 65° C. for 20 minutes (curve O——O).

The results, given in Fig. 7, indicate that the heated thrombocytes showed some loss of activity when compared with fresh preparations with regard to the influence on the thrombin formation rate, but the yield of thrombin was identical in both series.

From these experiments it is concluded that thrombocytes, whether freshly prepared or frozen and thawed, or heated at 65° C. for 20 minutes, are active components of the thromboplastin complex if present in adequate concentration.

*The effect of plasma components on the rate of formation and yield of thrombin.*

Normal blood or plasma is given intravenously for the correction of certain haemorrhagic conditions. This is usually carried out empirically, since the capacity of a given volume of normal blood to correct a deficiency has not been established. The capacity of normal blood to correct such deficiencies was studied by the addition of varying volumes of Ba-plasma as a source of $\alpha$-thromboplastin, or of stored plasma as a source of $\beta$-thromboplastin, to diluted blood.

TABLE 2.

*Influence of $\alpha$- or $\beta$-prothromboplastin on the rate of formation and yield of thrombin in shed blood.*

| | Blood vol. in ml. | Thromboplastin addition $\alpha$- | $\beta$- | Maximum thrombin formation minutes | units |
|---|---|---|---|---|---|
| A | 0·04 (14 cases) | — | — | 15·8 (14–18) | 0·95 (0·85–1·1) |
| | 0·04 (14 cases) | 0·005 | — | 15·5 (12–18) | 0·94 (0·85–1·1) |
| B | 0·02 | — | — | 24 | 0·42 |
| | 0·02 | 0·02 | — | 18 | 0·41 |
| C | 0·02 | — | — | 20 | 0·52 |
| | 0·02 | — | 0·04 | 20 | 1·15 |
| D | 0·02 | — | — | 25 | 0·57 |
| | 0·02 | — | 0·04 | 19 | 1·4 |
| E | 0·04 | — | — | ∞ | 0·0 |
| | 0·04 | 0·005 | — | 22 | 0·66 |
| F | 0·04 | — | — | ∞ | 0·0 |
| | 0·04 | — | 0·005 | 18 | 1·1 |

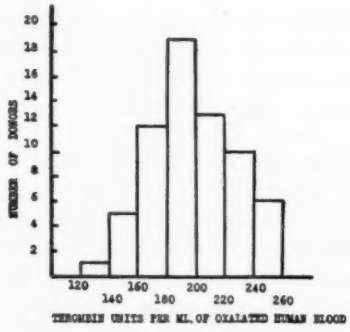
Experimental conditions were similar to those given in (2) Fig. 1. Reaction mixtures were made up with 0·15 M-sodium chloride to a total volume of 1·8 ml. The thrombin results are calculated for 0·2 ml. of the reaction mixture.

The results given in A, Table 2, refer to average figures of fourteen specimens of normal human blood. The addition of 12·5 p.c. normal Ba-plasma, which contains $\alpha$-prothromboplastin, did not influence the yield of thrombin, nor was the reaction time shortened. In B, equal amounts of $\alpha$-prothromboplastin added to normal blood showed some activation indicated by the shortening of the reaction time. These observations indicate that normal blood contains an adequate quantity of $\alpha$-prothromboplastin for the conversion of prothrombin to thrombin. Experiments recorded under C and D show that the prothrombin content of 0·04 ml. plasma, stored for four weeks, which was free of $\alpha$-prothromboplastin, was converted into thrombin by 0·02 of normal fresh blood. Assuming that this contained approximately 0·01 ml. plasma, it means that one volume of normal blood can form sufficient thromboplastin to convert the prothrombin

content of four volumes of blood deficient in α-prothromboplastin. Additional evidence for the presence of an excess of thromboplastin precursors in normal blood was obtained in experiments E and F. Blood for experiment E was taken from a male bleeder who had a complete deficiency of α-prothromboplastin (haemophilia). Under the above experimental conditions no thrombin was formed from his diluted blood during the incubation period of 60 minutes. The addition of 12·5 p.c. Ba-plasma resulted in a significant thrombin production in 22 minutes. For experiment F blood was taken from a male bleeder with complete absence of β-prothromboplastin deficiency. It was found that the addition of 12·5 p.c. stored plasma corrected the deficiency.

These results suggest that normal blood has approximately a four-fold excess of α- and β-prothromboplastin.

As it is intended to study the rate of formation and yield of thrombin in pathological conditions, it is necessary to find out first the normal variation in rate of formation and yield of thrombin. The following technique was used: 0·5 ml. of venous oxalated blood was added to 2 ml. 0·15 M-sodium chloride within two minutes of the blood collection. 0·2 ml. of this dilution was mixed with a solution consisting of 0·6 ml. of 0·025 M-calcium chloride, 0·7 ml. 0·15 M-sodium chloride and 0·3 ml. 0·18 M-pyrocatechol in veronal buffer pH 7·4 at 28° C. At intervals beginning ten minutes after the start, 0·2 ml. reaction mixture was removed and blown into a mixture of 0·2 ml. human Ba-plasma and 0·05 ml. 0·18 M-pyrocatechol in veronal buffer kept at 28° C. The determinations of thrombin were carried out until two successive results were identical. The clotting times were converted into thrombin units which, multiplied by 225, gave the quantity per ml. of oxalated blood. The following histogram indicates the maximum thrombin yield in normal human blood as determined by the outlined technique.




The histogram was prepared from 66 blood specimens obtained from healthy donors of either sex aged 20–53 years. Maximum thrombin formation occurred in 14 to 19 minutes.

## DISCUSSION.

The presented results indicate that the addition of pyrocatechol to diluted blood which is in the process of clotting allows quantitative measurement of the thrombin formed. It was observed that under these conditions the amount of thrombin was identical with that obtainable in the presence of brain thromboplastin. It appears, therefore, first that pyrocatechol was active in counteracting anti-thrombin, and secondly, that the thromboplastin complex produced during clotting was adequate for complete conversion of prothrombin into thrombin. No evidence for interference of pyrocatechol with any clotting components has been obtained. It is therefore believed that the determination of the rate of formation and yield of thrombin in the presence of pyrocatechol does not produce a distorted picture of the clotting process. The study of the formation and yield of thrombin was carried out in order to obtain information about the role of the several blood thromboplastin components in haemostasis. Although it is realised that this function is partly dependent on blood activity and partly on vascular properties, this discussion is only centred around blood factors.

The presented experiments have given support to the assumption that at least two plasma precursors, calcium ions and thrombocytes, are required for adequate blood thromboplastin activity. Certain physico-chemical properties of the surface with which the precursors come in contact are also essential for this activation. In the absence of thrombocytes, clotting can take place as, for instance, in lymph, but the amount of thromboplastin formed is only sufficient to convert a fraction of prothrombin into thrombin. In cases of severe thrombocytopenia, sufficient thromboplastin is produced from the plasma factors, but thrombin formation is delayed to an extent which makes efficient haemostasis impossible. The experiments have shown that the thrombocytes do not contain active thromboplastin, because adequate numbers of them in the absence of either $\alpha$- or $\beta$-prothromboplastin did not produce any thrombin in diluted blood. This deficiency, however, could be repaired by the supply of the missing plasma factor. The mechanism of prothrombin activation by blood thromboplastin is different from that of brain as indicated by the different rates of thrombin formation in the two cases. This is due to the fact that blood thromboplastin has to be formed from precursors, a complex reaction which requires time, whereas brain thromboplastin is preformed and can react immediately with the prothrombin complex. The role of the thrombocytes in the process of thromboplastin formation seems to be of a catalytic nature, due to a specific—relatively heat stable—property of the thrombocytes. Regarding the capacity of the thromboplastin precursors in plasma to convert prothrombin, it was found that normal blood has at least a four-fold excess of potential thromboplastin. It would appear that a bleeding tendency could occur at plasma levels below 25 p.c. of each of the plasma thromboplastin precursors.

The quantitative determination of the blood thromboplastin components should give an indication of haemostatic efficiency. This is, of course, of clinical interest. Whereas gross deficiencies are usually readily recognized, slight deficiencies often pose a problem. A definite answer is of importance to the patient and since thromboplastin deficiencies can be inherited, eugenic implications have to be considered. It is, therefore, essential to decide on the basis of laboratory tests whether a person is normal or a bleeder, or whether the haemorrhagic tendency is due to other causes. No specific reactions are known for thromboplastic substances except their ability to convert prothrombin into thrombin. Of the existing tests whole blood clotting time, and the serum prothrombin determination should be mentioned in this connection. These tests have given useful information if the results deviate markedly from the normal value, but when they fall within the normal range a haemorrhagic tendency is not excluded as clinical experience has shown (Merskey, 1951; Quick and Hussey, 1952; Graham *et al.*, 1953).

The relative insensitivity of the existing tests is due to their static nature, but clotting is a dynamic process in which a number of components take part. The several reactions which take place have different reaction rates, and haemostasis is characterized by the reaction proceeding with the slowest reaction rate. Therefore it seems that the determination of the rate of thrombin formation in shed blood might give information of the haemostatic condition of a person, which could not be supplied by the determination of any single factor. MacFarlane and Biggs (1953) have pointed out that "more information would be provided if a series of determinations of the thrombin content of whole blood were made at intervals during clotting." The thrombin generation tests of these workers is carried out in undiluted blood. Pitney and Dacie (1953) used citrated plasma. Admittedly whole blood reflects closer the events occurring in the circulation, but the tests are of a qualitative nature because the amount of thrombin determined is only a fraction of the thrombin production, due to the unimpaired activity of plasma antithrombins. It was thought of advantage to use diluted blood and add pyrocatechol, which counteracts antithrombin. This is of value since the antithrombic activity of plasma specimens varies and so excludes a quantitative comparison in the absence of pyrocatechol. The produced thrombin is taken as a measure of the activity of the plasma thromboplastin complex. Further, diluted blood allows the addition of compounds whose thromboplastic activity can be measured, and if a shortening of the rate of thrombin formation by the added compound has taken place, this would indicate a deficiency of the added factor in the original specimen. The diagnosis of an $\alpha$- or $\beta$-prothromboplastin deficiency and an assay procedure can readily be carried out in this way.

It was shown that under the experimental conditions used, maximum thrombin formation in normal diluted blood occurred between 14–19 minutes with a yield of 120–260 units of thrombin per ml. of oxalated blood. A prolongation

of the reaction time and a diminution of thrombin yield is considered as pathological. It will be necessary to show whether the test is of value in haemorrhagic conditions of a mild nature. Such an investigation has been carried out in connection with clinical investigations. The result of this work will be presented elsewhere.

## SUMMARY.

Thrombin produced in diluted clotting blood can be measured quantitatively in the presence of pyrocatechol.

Arterial and venous human blood produce thrombin at the same rate and yield.

In diluted capillary blood thrombin is produced at a faster rate than in systemic blood, due to the presence of tissue thromboplastin, but the thrombin yield is identical in capillary and systemic blood.

Maximum blood thromboplastin activity is obtained from a system containing at least two plasma thromboplastic components and adequate concentration of thrombocytes.

Fresh thrombocytes show higher activity than stored or frozen and thawed or heated thrombocytes.

Normal blood has at least a four-fold excess of potential thromboplastin.

The rate of formation and yield of thrombin produced in diluted blood in the presence of pyrocatechol is an indication of a person's haemostatic efficiency.

The physiological variation of the rate of formation and yield of thrombin in diluted blood is presented.

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# THE KINETICS OF THE REACTIONS CATALYZED BY ACONITASE

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Since the discovery of aconitase by Martius and Knoop (1937), it has been generally accepted that citric acid is transformed into *iso*citric acid via *cis*-aconitic acid. However, Martius and Lynen (1950) and Friedrich-Freska and Martius (1951) claimed that there was a direct conversion of citric acid to *iso*citric acid, as they did not observe a latent period at the start of the reaction. Further, as a result of the mathematical treatment of the reaction velocities, Friedrich-Freska and Martius (1951) supposed that after combination of the substrate with the enzyme, there is formed an intermediate complex which can decompose into enzyme-*cis*-aconitic acid, enzyme-*iso*citric acid or enzyme-citric acid. They regarded *cis*-aconitic acid as being only a by-product of the aconitase reaction. These authors also studied the six initial reaction velocities associated with aconitase activity and claimed that the difference in the rates could account for the transient accumulation of *iso*citric acid above the equilibrium value that occurs when the reaction is started with *cis*-aconitic acid. On account of the agreement between their experimental findings and mathematical predictions, they concluded that a single enzyme could catalyse the conversion of each of the three acids to the other two.

The mathematical treatment which Friedrich-Freska and Martius (1951) applied to their results has been strongly criticised by Kacser (1952). Krebs and Holzach (1952) re-investigated the problem of the lag period in the conversion of citric acid to *iso*citric acid, and showed that it did occur.

As the studies on the kinetics of aconitase had been made with crude or partially purified preparations of the enzyme, the previous work was repeated with a more highly purified enzyme preparation (Morrison, 1954a). The question of the lag period was investigated, also the relative rates of the reactions catalyzed by aconitase. The Michaelis constants of aconitase for citric, *cis*-aconitic and *iso*citric acids were determined. For a preliminary communication see Morrison (1954b).

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## Materials and Methods.

*Estimation of* cis-*aconitic acid.*

*cis*-Aconitic acid was estimated in the first place by the method of Dickman (1952), which was based on the reduction of potassium permanganate by a compound containing a double bond, in the presence of metaphosphoric acid. The results indicated that there was an initial rapid formation of *cis*-aconitic acid from *iso*citric acid which was followed by a slower production of *cis*-aconitic acid. Moreover, there was no relationship between the amount of enzyme added and the formation of *cis*-aconitic acid during the initial stages of the reaction. Investigation showed that the addition of the enzyme to a mixture of buffer, *iso*citric acid and metaphosphoric acid (which was used to stop the reaction) gave rise to a compound which was capable of reducing the potassium permanganate and which thus was estimated as *cis*-aconitic acid. The amount of this compound which formed rose with increasing amounts of enzyme, although the increase was not linear. If this blank value were subtracted from the subsequent estimations, a linear rate was obtained which was proportional to the amount of enzyme added.

A similar phenomenon was encountered when the formation of *cis*-aconitic acid from citric and *iso*citric acids was determined in a Beckman spectrophotometer at a wavelength of 240 m$\mu$ by the method of Racker (1950). Although neither the enzyme nor the other components of the system gave any appreciable absorption, on mixing there was a rapid increase in the extinction coefficient. With *iso*citric acid as the substrate, this rapid increase in the extinction coefficient continued over a period of 1 minute, after which the reaction rate became linear. Fig. 1 shows what happens on adding the enzyme to a mixture of buffer and citric acid. The concentration of citric acid used for curves 1, 2 and 3 affects both the point where the rapid increase in the absorption ceases and the time for that point to be reached, but the subsequent linear rates of the reactions are the same. With lower concentrations of citric acid, a smaller increase in the extinction coefficient is obtained before the reaction rates become linear.

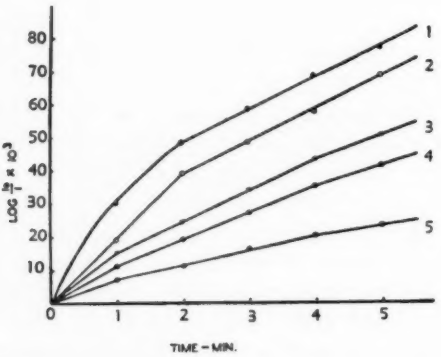



**Fig. 1.** The enzymic and non-enzymic reaction rates on adding aconitase activated with $Fe^{2+}$ and cysteine to various concentrations of citric acid. The system contained 0·05 M phosphate buffer (pH 7·7), 8 $\mu$g. of the aconitase preparation and the following amounts of citric acid:

1. $5\times10^{-2}$ M 2. $2\times10^{-2}$ M 3. $1\times10^{-2}$ M 4. $5\times10^{-3}$ M 5. $1\times10^{-3}$ M

The enzyme used in these experiments had been activated with $Fe^{2+}$ and cysteine. As the concentration of the enzyme was small, it seemed more likely that the rapid increase in the absorption was due to the interaction of the $Fe^{2+}$ with the substrates rather than to the interaction of the substrates with the enzyme. On adding $Fe^{2+}$ and cysteine to both citric and *iso*citric acids in the same concentrations in which they were present in the enzyme solution, identical rapid increases in the extinction coefficients were obtained. At the point where the reaction became linear in the presence of the enzyme, the non-enzymic reaction ceased. If these values were subtracted from those obtained in the presence of the enzyme, the rates were linear from the start of the reaction. In view of this finding, it was important to distinguish the non-enzymic from the enzymic reaction rate. The method of Racker (1950) was used for following the production of *cis*-aconitic acid in the experiments reported in this paper, and only the enzymic reaction rates have been plotted in the graphs.

*Estimation of citric and isocitric acids.*

Citric acid was estimated by the method of Pucher, Sherman and Vickery (1936) as modified by Buffa and Peters (1949). *iso*citric acid was estimated by the method of Ochoa (1951). As the final aconitase preparation contained *iso*citric dehydrogenase, a non-activated solution was used as the source of this enzyme. The aconitase activity of this solution was so small that there was no detectable difference in the reaction rate when it was added to a medium containing the activated enzyme.

*Activation of aconitase.*

Aconitase was prepared and activated with $Fe^{2+}$ and cysteine before use by the methods previously described (Morrison, 1954a). The enzyme solutions contained 32 $\mu$g. of the final aconitase preparation/ml. in the presence of $5\times10^{-4}$ M $Fe^{2+}$ and $10^{-2}$ M cysteine.

*Reagents.*

Coenzyme II was prepared by the method of LePage and Mueller (1949) and had a purity of 48 p.c. The other reagents were the same as described in the previous paper (Morrison, 1954c).

## RESULTS.

### *Relative reaction rates.*

The relative rates of the six reactions catalyzed by aconitase are shown in Fig. 2 and a diagrammatic representation of these rates is shown in Fig. 3. These results agree reasonably well with those obtained by Friedrich-Freska and Martius (1951), with the exception of the reaction citric acid→*iso*citric acid. The rate of this reaction was found to be one third of that reported by these authors. It will be noted that the rates in the case of the reactions *iso*citric acid→citric acid and citric acid→*iso*citric acid are linear only after 5 minutes. It was these rates which were taken to be the initial rates of the reactions as quoted in Fig. 3, although, strictly speaking, they are not initial rates. When the lines representing the rates of these reactions are produced backwards, they cut the time axis, which indicates that there is an initial latent period. Thus, the conversions must occur via an intermediate substance which is most likely *cis*-aconitic acid, as no other intermediate substance is known.

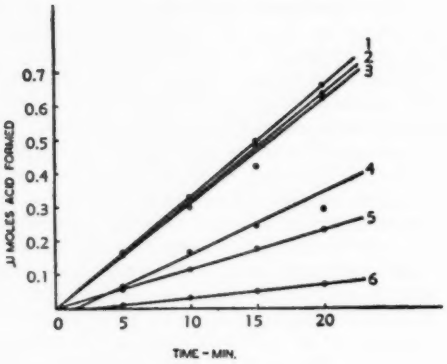



**Fig. 2.** The relative rates of the reactions catalyzed by aconitase. The reaction mixture contained 0·05 M phosphate buffer (pH 7·7), together with either *cis*-aconitic acid ($5\times10^{-3}$ M), *iso*citric acid ($5\times10^{-3}$ M) or citric acid ($5\times10^{-3}$ M). Total volume 3·0 ml., temperature 22° C. Reactions 2 and 6 were stopped at 5-minute intervals by the addition of 0·3 ml. of N HCl. The solutions were neutralized by the addition of 0·3 ml. of N NaOH before aliquots were taken for the estimation of *iso*citric acid. Reactions 3 and 4 were stopped at 5-minute intervals by the addition of 0·5 ml. of 50 p.c. (w/v) trichloroacetic acid and samples were analyzed for citric acid. The formation of *cis*-aconitic acid was determined directly in the Beckman spectrophotometer, readings being taken at 1-minute intervals. A solution containing 0·1 μmol. of *cis*-aconitic acid/ml. gave an extinction coefficient of 0·400. The amount of enzyme solution used in each test is given in brackets.

1. *iso*citric acid→*cis*-aconitic acid (0·1 ml.)
2. *cis*-aconitic acid→*iso*citric acid (0·1 ml.)
3. *cis*-aconitic acid→citric acid (0·4 ml.)
4. *iso*citric acid→citric acid (0·4 ml.)
5. citric acid→*cis*-aconitic acid (0·2 ml.)
6. citric acid→*iso*citric acid (0·5 ml.)

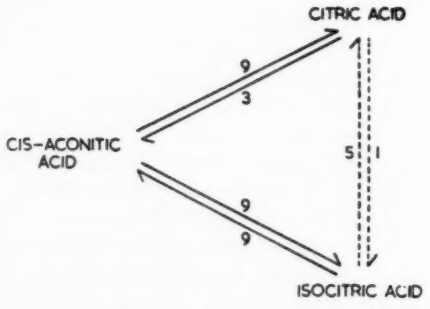



**Fig. 3.** The relative rates of the reactions catalyzed by aconitase. A diagrammatic representation. The experimental conditions were as described in Fig. 2.

If higher concentrations of aconitase are used to study the conversion of citric acid to *iso*citric acid and *vice versa*, the latent period is decreased to such an extent that the rate of formation of product is virtually linear. This fact was utilized in previous investigations (Morrison, 1954c). It also possibly explains why Martius and Lynen (1950) and Friedrich-Freska and Martius (1951) failed to observe a latent period in the above reactions.

*Determination of the lag phase.*

Fig. 4 illustrates the lag period of the reaction citric acid→*iso*citric acid in more detail. The formation of *iso*citric acid was studied by measuring the reduction of coenzyme II in the presence of *iso*citric dehydrogenase. The pH

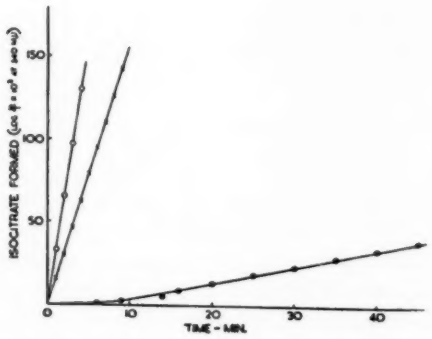



**Fig. 4.** Time curves for the formation of *iso*citric acid from *cis*-aconitic and citric acids. The system contained 0·05 M phosphate buffer (pH 7·2), 1·8 μmol. of $MnCl_2$, 0·135 μmol. of CO II, 3 μg. of the activated aconitase preparation, 16 μg. of the non-activated aconitase preparation (*iso*citric dehydrogenase), together with *iso*citric acid ($5\times10^{-3}$ M), *cis*-aconitic acid ($5\times10^{-3}$ M) or citric acid ($5\times10^{-3}$ M). Total volume 3·0 ml., temperature 22° C.

O=*iso*citric acid. X=*cis*-aconitic acid. ●=citric acid.

in this instance was 7·2 rather than 7·7. The reason for this alteration was that it was not possible to study the reduction of coenzyme II by *iso*citric dehydrogenase in the presence of phosphate buffer at pH 7·7 on account of the precipitation of manganese. The oxidation of *iso*citric acid was the fastest reaction, so that this was a valid means of estimating the rate at which *iso*citric acid was formed from citric and *cis*-aconitic acids. It can be seen that there is no lag period in the conversion of *cis*-aconitic acid to *iso*citric acid, whereas the lag period is marked with citric acid as the substrate; there was in fact no measurable amount of *iso*citric acid formed until 6 minutes after the start of the reaction. These findings are in agreement with those of Krebs and Holzach (1952).

*Effect of the simultaneous addition of* isocitric acid *and citric acid on the rate of formation of* cis-*aconitic acid.*

In an endeavour to determine whether or not the same enzyme centre(s) was concerned in the formation of *cis*-aconitic acid from both citric and *iso*citric acids, the rate of *cis*-aconitic acid formation was determined in the presence of each acid separately, and in the presence of both acids. So that the results should not be complicated by the different affinities of the enzyme for the two acids, they were added in a concentration ten times greater than their Michaelis constants. (The actual Michaelis constant values are reported below.)

TABLE 1.

*Influence of citric acid on the rate of the reaction* isocitric *acid to* cis-*aconitic acid.*

(The reactions were carried out in the presence of 0·05 M phosphate buffer (pH 7·7), 5 μg. of the aconitase preparation, $3{\cdot}6\times10^{-2}$ M citric acid and/or $4{\cdot}8\times10^{-2}$ M *iso*citric acid. Total volume 3·0 ml., temperature 22° C.)

| Substrate | Reaction rate ($\Delta\epsilon$/min.$\times10^3$) |
|---|---|
| Citric acid | 15 |
| *iso*Citric acid | 45 |
| Citric acid+*iso*citric acid | 29 |

The results of Table 1 show that the rate of the conversion of *iso*citric acid to *cis*-aconitic acid is influenced by the presence of citric acid, in fact the rate in the presence of both acids is equal to half the combined rates obtained with each acid separately. This result would seem to indicate that the same centre(s) is concerned in the two reactions. In a more extensive investigation Tomizawa (1953) reached a similar conclusion.

*Determination of Michaelis constants.*

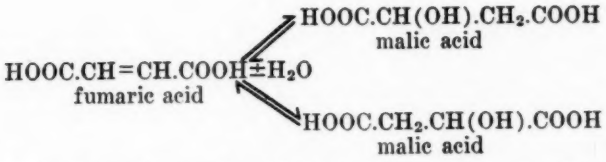
The Michaelis constant of aconitase for citric acid was found to be $3{\cdot}6\times10^{-3}$ M, for *cis*-aconitic acid $1{\cdot}2\times10^{-4}$ M, and for D-*iso*citric acid $4{\cdot}8\times10^{-4}$ M at pH 7·7 and 22° C. in the presence of 0·05 M phosphate buffer. Racker (1950) found that the Michaelis constant of aconitase for citric acid was $1{\cdot}1\times10^{-3}$ M and for D-*iso*citric acid $4\times10^{-4}$ M at pH 7·4 and 25° C., whilst under the same conditions Tomizawa (1953) has reported the following Michaelis constant values: citric acid, $9\times10^{-4}$ M; *cis*-aconitic acid, $9\times10^{-5}$ M, and D-*iso*citric acid $3{\cdot}2\times10^{-4}$ M. Racker (1950) did not measure the Michaelis constant of aconitase for *cis*-aconitic acid. Thus, the affinity of aconitase for *cis*-aconitic acid is greater than for *iso*citric or citric acids.

The Michaelis constant of aconitase for *cis*-aconitic acid was determined from the reaction *cis*-aconitic acid→citric acid. It would have been of interest to determine the value also from the reaction *cis*-aconitic acid→*iso*citric acid, for

if they were the same it would have been strong evidence in favour of the idea that both reactions are catalyzed at the same centre(s) of the same enzyme. Unfortunately, the rate of *iso*citric acid formation could not be studied at pH 7·7 with *iso*citric acid dehydrogenase and coenzyme II in the presence of phosphate buffer for reasons previously mentioned. Attempts were made to determine the amount of *iso*citric acid present in samples of the reaction mixture after various periods of time, but reliable results could not be obtained on account of the small amounts of *iso*citric acid formed from low concentrations of *cis*-aconitic acid.

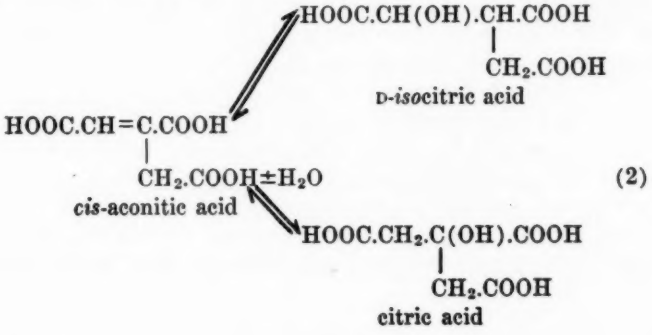
## DISCUSSION.

Aconitase has been considered as an unusual enzyme in so far as it has three substrates which are interconvertible. However, as pointed out by Ogston (1951), there is no reason why it should not be considered in a broad sense as being similar to fumarase. The reaction catalyzed by fumarase can be written as follows:




(1)

Theoretically, water can be added in two ways. (As L-malic acid is formed, the enzyme must be linked to the substrate so that the water molecules can approach from one direction only—otherwise D-malic acid would also be formed. The same reasoning applies in the case of aconitase as only D-*iso*citric acid is formed. Ogston (1951) has proposed that the linkage of *cis*-aconitic acid to the enzyme involves a three-point attachment in order to explain this fact.) On account of fumaric acid being a symmetrical molecule, the same compound is formed irrespective of the way in which the water molecules are added across the double bond of fumaric acid. Aconitase can be considered as catalyzing essentially the same reaction which can be written in a similar fashion:




(2)

As *cis*-aconitic acid is an asymmetrical molecule, the products formed as a result of the addition of a water molecule across the double bond can be distinguished.

Citric acid formed as a result of the condensation reaction between acetyl-coenzyme A and oxalacetic acid is further metabolized by conversion to *cis*-aconitic acid and *iso*citric acid as shown by Krebs and Holzach (1952) and confirmed here. As the reaction is usually written

citric acid→*cis*-aconitic acid→*iso*citric acid ..... ..... (3)

there has been a tendency to think of the conversion of citric acid to *iso*citric acid as two separate reactions possibly catalyzed by two distinct enzymes. The experimental results of Morrison (1954a; 1954c) and Buchanan and Anfinsen (1949) indicate that aconitase activity is associated with only a single species of protein molecule. When the reaction is written as shown in equation (2), the conversion of citric acid to *iso*citric acid by a single enzyme need not be considered unusual. Both citric and *iso*citric acids can be thought of as being substrates of aconitase, that is, the enzyme does not have an absolute specificity. The reaction rates and the affinity of the enzyme for each acid differ and thus support this contention. Whereas with other enzymes not having an absolute specificity, the product of the reaction varies according to the substrate added, with aconitase the product of the reaction with each substrate is *cis*-aconitic acid. Because of this and the fact that aconitase is a catalyst, the reverse reactions must also occur, and so citric acid can be converted to *iso*citric acid. This reaction must be then considered as being due to the establishment of an equilibrium rather than as a direct conversion of one acid to the other.

If the above concept is correct, two conditions must hold; citric acid must be converted to *iso*citric acid *via cis*-aconitic acid, and the same catalytic centre(s) must be concerned in all the reactions catalyzed by aconitase. With regard to the first point, the results of Krebs and Holzach (1952), and those reported here, indicate that citric acid is converted to *iso*citric acid *via cis*-aconitic acid. Thus the intermediate complex of Friedrich-Freska and Martius (1951) can be considered as being nothing more than the complex formed between aconitase and *cis*-aconitic acid. No experimental evidence has been previously presented to indicate that only one catalytic centre of the enzyme is concerned with all the reactions. The results obtained here can be interpreted to show that this is the case. It was found that citric acid was converted to *cis*-aconitic acid at a rate which can be arbitrarily called 1, whilst *iso*citric acid was converted to *cis*-aconitic acid at a rate of 3. If two different centres were concerned in these reactions, it would be expected that the simultaneous addition of both *iso*citric acid and citric acids would give a rate greater than 3, theoretically 4, as a result of summation. If only one centre were concerned, the rate should be equal to half the combined rate of each reaction. That is, there would be an equal

chance of either acid reacting with the active centre(s) of enzyme. The results showed that the latter is actually what happens, for the rate in the presence of both substrates is 2. Another possible explanation must also be borne in mind. If two centres were concerned and the reaction taking place at each was inhibited 50 p.c. by the other substrate, the reaction rate in the presence of both substrates would be 2. However, other evidence, particularly the pH-activity curves obtained for three of the reactions in different buffers, favours the idea of one centre (Morrison, 1954c).

Friedrich-Freska and Martius (1951) explained the transient accumulation of *iso*citric acid above the equilibrium value when the reaction was started with *cis*-aconitic acid as being due to the difference in the initial reaction rates. The same reasoning was applied to explain the transient accumulation of *cis*-aconitic acid when the reaction was started with *iso*citric acid. As the rates found here are essentially the same as those of Friedrich-Freska and Martius, this explanation seems tenable. Although these authors claimed that there was no lag period with the reactions citric acid→*iso*citric acid and *iso*citric acid→citric acid, the validity of the argument is not affected, as only the four true initial reaction rates are concerned. The rates of the reactions citric acid→*iso*citric acid and *iso*citric acid→citric acid are consistent with the idea that they are the sum of two reactions, i.e., the sum of the reactions citric acid→*cis*-aconitic acid and *cis*-aconitic acid→*iso*citric acid and *vice versa*.

Martius and Leonhardt (1943) found that *cis*-aconitic acid was converted to both *iso*citric acid and citric acid at equal rates, whereas Friedrich-Freska and Martius (1951) found that *cis*-aconitic acid was converted to *iso*citric acid at almost 1·5 times the rate at which it was converted to citric acid. The results obtained here agree with those of Martius and Leonhardt (1943). The fact that *cis*-aconitic acid is converted to both *iso*citric and citric acids at an equal rate raises an interesting point. Ogston (1951) analyzed the aconitase reactions theoretically and concluded that when *cis*-aconitic acid was linked to the enzyme the water molecules of the medium have an equal chance of reacting to form either citric acid or *iso*citric acid. He quotes the results of Martius and Leonhardt (1943) in support of this idea. Kacser (1952) has also concluded from a mathematical treatment of the reactions catalyzed by aconitase that the water molecules approach from solution.

At present there is no experimental evidence that the water molecules concerned in the reaction approach the enzyme from the medium. It is also possible that they could react with *cis*-aconitic acid by prior absorption on the enzyme surface. Under these circumstances it might be expected that the enzyme would have an orientating influence on the water molecules so that citric acid would be formed in preference to *iso*citric acid or *vice versa*. But the finding that the rates are equal is inconsistent with this idea. On the other hand, if the water molecules approached *cis*-aconitic acid linked to the enzyme from

the medium, it might also be expected that the acid itself, being an asymmetric molecule, would influence the way in which the water molecules would be added across the double bond. The only way in which the finding that *cis*-aconitic acid is converted to both citric and *iso*citric acid at an equal rate can be explained, is that the effect of the enzyme on *cis*-aconitic acid is to eliminate the normal directional influence of *cis*-aconitic acid on the water molecules approaching from solution.

## SUMMARY.

It was shown that $Fe^{2+}$ and cysteine react with citric and *iso*citric acids so that the products estimate as *cis*-aconitic acid.

The relative rates of the reactions catalyzed by aconitase have been determined.

A definite lag period was shown to occur in the conversion of citric acid to *iso*citric acid.

The rate of formation of *cis*-aconitic acid from citric acid plus *iso*citric acid was shown to be an equal to half the sum of the rates of *cis*-aconitic-acid formation from each acid separately.

The Michaelis constants of aconitase for citric, *cis*-aconitic and *iso*citric acids were determined.

Some aspects of aconitase action have been discussed.

*Acknowledgments.* The author wishes to express his thanks to Sir Rudolph Peters, F.R.S., Dr. R. B. Fisher and Dr. A. G. Ogston for their advice and interest during the course of this work. Thanks are also due to Miss S. Read for skilled technical assistance.

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# THE INFLUENCE OF BUFFERS ON THE pH OPTIMUM OF ACONITASE

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*(Accepted for publication 28th June, 1954.)*

Jacobsohn (1941) found that the pH optimum of aconitase in the presence of phosphate buffer was in the neutral region. A more extensive study of the influence of pH on aconitase activity was made by Johnson (1939). He claimed that the pH-activity curve showed a sharp maximum at pH 7·4, but this value must be regarded as approximate. The pH values chosen for testing the aconitase activity were widely spaced, and no mention was made of the buffers used to cover the extensive pH range.

The above information was gained from studies with crude aconitase preparations. Aconitase has now been highly purified (Morrison 1954a) and shown to be dependent on the presence of $Fe^{2+}$ and a reducing agent for maximum activity (Dickman and Cloutier, 1951; Morrison, 1954a). A more detailed investigation of the effect of pH on the activated, purified enzyme showed that the pH optimum is dependent on the type of buffer used. For a preliminary report of this work, see Morrison (1954b).

## Materials and Methods.

*Determination of aconitase activity.*

Aconitase was activated with $Fe^{2+}$ and cysteine by the method previously described (Morrison, 1954a). The enzyme solution contained 60 $\mu$g. of the final aconitase preparation/ml. in the presence of $5\times10^{-4}$ M $Fe^{2+}$ and $10^{-3}$ M cysteine. The veronal acetate buffer was prepared according to Michaelis (1931), whilst the other buffers were 0·1 M. 0·15 ml. samples of the enzyme solution were added to media containing 2·5 ml. of the appropriate buffer, 0·5 ml. of *cis*-aconitic acid or *iso*citric acid (final concentration $4\times10^{-3}$ M) and 1·85 ml. of water. The reaction was stopped after 15 min. at 30° C. by the addition of 0·5 ml. of 50 p.c. (w/v) trichloroacetic acid when citric acid was determined, or 0·5 ml. of 10 p.c. (w/v) metaphosphoric acid when *cis*-aconitic acid was estimated.

*Determination of citric and* cis-*aconitic acids.*

Citric acid was estimated by the method of Pucher, Sherman and Vickery (1936) as modified by Buffa and Peters (1949), whilst *cis*-aconitic acid was estimated by the method of Dickman (1952).

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*Reagents.*

*cis*-aconitic anhydride was prepared by the method of Malachowski and Maslowski (1928). Sodium *cis*-aconitate was formed by neutralizing the anhydride to pH 7·4 with N-NaOH DL-*iso*citric acid was prepared by hydrolysis of the lactone. Trichloromethylparaconic acid was prepared by the method of Fittig and Miller (1889) and converted into *iso*citric lactone by the method of Krebs and Eggleston (1944). Sodium *iso*citrate was formed by hydrolysis of the lactone with NaOH as described by Krebs and Eggleston (1944). Both acids were prepared by Mr. R. W. Wakelin. L-Cysteine hydrochloride was obtained from Roche Products, Ltd., and was free from iron. N-Ethylmorpholine was kindly supplied by Professor A. Albert. Veronal, glycerophosphoric acid, glycylglycine, boric acid and metaphosphoric acid were British Drug Houses, Ltd., Laboratory Reagents. The other reagents were A.R.

## RESULTS.

*Aconitase activity in the presence of different buffers at pH 7·4.*

It has previously been reported (Morrison 1954a) that the inactivating effect of various buffers could be eliminated if the enzyme were added to the buffer-substrate mixture. These activity tests were carried out in that fashion. Fig. 1 shows the effect of six different buffers at pH 7·4 on the activity of aconi-

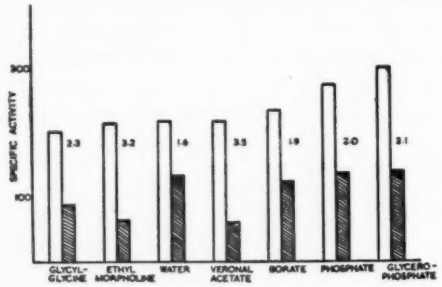


**Fig. 1.** The activity of aconitase in the presence of different buffers at pH 7·4. Enzyme activity determined as described in the text. Temperature 30° C., buffer 0·05 M.
Unshaded bars=*cis*-aconitic acid→citric acid .... .... .... .... (1)
Shaded bars=*iso*citric acid→citric acid .... .... .... .... .... (2)
Figures indicate the ratio of reaction (1) to reaction (2).

tase as compared to the activity when the reactions were carried out in the absence of buffer. For comparative purposes, the reactions *iso*citric acid→citric acid and *cis*-aconitic acid→citric acid were studied. It is clear that the composition of the buffer has a profound influence on these reactions. In the reaction *cis*-aconitic acid→citric acid, the enzyme activity in glycylglycine is decreased as compared to the activity in the absence of buffer; veronal acetate and N-ethylmorpholine have no effect, whilst borate, phosphate and glycerophosphate increase the activity. In the reaction *iso*citric acid→citric acid, the activity is approximately the same in phosphate and glycerophosphate, and in the absence of buffer, whereas the activity is lower in borate, glycylglycine, N-ethylmorpholine and veronal

acetate. The consequence is that the ratio of the rates of the two reactions varies according to the buffer used.

There seemed to be two possible explanations for the above results; that the various buffers have different abilities to activate and inactivate the two reactions, or that the pH optimum or optima of the reactions are influenced by the ions present in solution. As the latter explanation seemed more likely, the pH optimum of the enzyme in catalyzing both reactions was determined in the presence of different buffers.

*Influence of buffers on the pH optimum of aconitase.*

It can be seen from Figs. 2 and 3 that the pH optimum of aconitase is influenced by the buffer in which the reaction is carried out. The following pH optima were found for both the reactions: glycerophosphate, 7·5; phosphate, 7·7; N-ethylmorpholine, 8·1, and veronal acetate 8·6. The absolute rates of each reaction were approximately the same at the pH optimum for each buffer, so that the ratio of the rates of the reactions *cis*-aconitic acid→citric acid to *iso*-citric acid→citric acid was about 1·8 in each case. This compares with values ranging from 1·6 to 3·5, which were obtained previously with these buffers at pH 7·4.

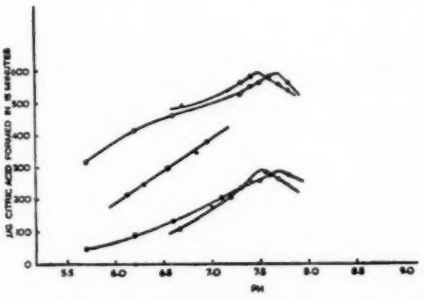

Fig. 2.

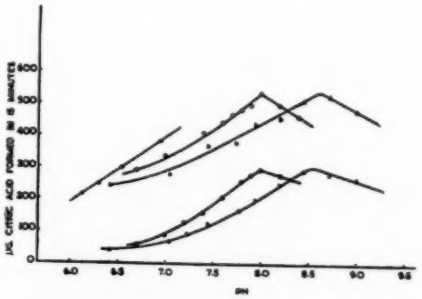

Fig. 3.

**Fig. 2.** Effect of buffers on the pH optimum of aconitase. Enzyme activities were determined as described in the text. Temperature 30° C., buffer 0·05 M.

×=no buffer
○=phosphate
△=glycerophosphate
} *cis*-aconitic acid→citric acid

●=phosphate
▲=glycerophosphate
} *iso*citric acid→citric acid

**Fig. 3.** Effect of buffers on the pH optimum of aconitase. Conditions the same as those of Fig. 2.

×=no buffer
○=N-ethylmorpholine
△=veronal acetate
} *cis*-aconitic acid→citric acid

●=N-ethylmorpholine
▲=veronal acetate
} *iso*citric acid→citric acid

It was not possible to determine the activity of aconitase over a wide range of pH in the absence of buffer on account of the poor buffering capacity of the aconitase substrates at higher pH values. However, the activity of the enzyme was determined for the reaction *cis*-aconitic acid→citric acid at the lower pH values. In Figs. 2 and 3 it is seen that over the pH range which could be studied the enzyme had not reached its maximum activity. If it is assumed that the optimum activity of the enzyme in the absence of buffer is the same as in the presence of buffers, and the line obtained is produced to the level of the optimal enzyme activity in the other buffers, it can be estimated that the pH optimum in the absence of buffer is about 8·0.

As the pH optimum of the reactions *cis*-aconitic acid→citric acid and *iso*-citric acid→citric acid was the same in the presence of a particular buffer and altered to the same extent in different buffers, it was of interest to determine if this were also the case for the reaction *iso*citric acid→*cis*-aconitic acid. The pH-activity curves obtained with phosphate and veronal acetate buffers, as shown in Fig. 4, indicate that this is indeed the case.

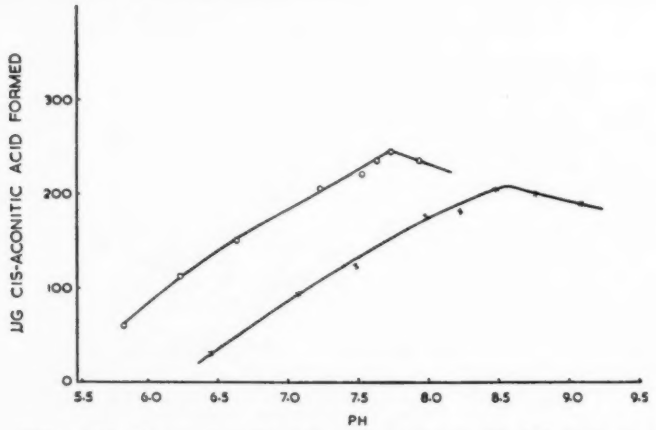


**Fig. 4.** Effect of buffers on the pH optimum of aconitase. Conditions the same as those described in Fig. 2 except that the reaction was stopped by the addition of 0·5 ml. of 10 p.c. (w/v) metaphosphoric acid.

O=phosphate
×=veronal acetate
} *iso*citric acid→*cis*-aconitic acid

*Inactivation of aconitase by buffers.*

Fig. 5 illustrates the rapid inactivation of aconitase that occurs when the enzyme is incubated in the absence of substrate for various periods of time with different buffers. The pH of each buffer was such that the aconitase activity was maximal. The rate of inactivation differs for each buffer and appears to be a function of the pH value.

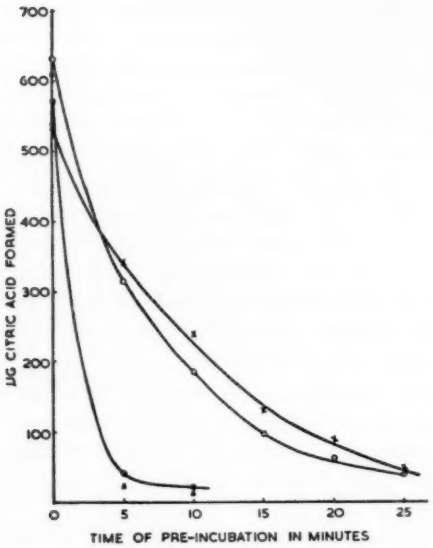


**Fig. 5.** Inactivation of aconitase by buffers in the absence of substrate. Aconitase activated with $Fe^{2+}$ and cysteine was incubated at 30° C. for various periods of time in the presence of 0·05 M buffer. *cis*-aconitic acid was added and the reaction stopped after 15 minutes. Solutions were analyzed for citric acid.

×=glycerophosphate (pH 7·5)
○=phosphate (pH 7·7)
●=N-ethylmorpholine (pH 8·1)
▲=veronal acetate (pH 8·6)

## DISCUSSION.

These results explain not only the different activities which were found in various buffers at pH 7·4, but also the apparent activation of aconitase by phosphate buffer at pH 7·4 as previously reported (Morrison, 1954a). The increases in enzyme activity are not due to the activation of the enzyme, but to the fact that the pH value chosen was closer to the optimum value in some buffers than it was in others. The alteration of the optimum pH of an enzyme is not a new phenomenon, but it is unusual to find that it is shifted to such an extent. Massey (1953) has reported similar effects on ions on the pH optimum of fumarase.

The mechanism by which ions influence the pH optimum of aconitase cannot be decided from the above results. Before any attempt is made to explain a particular pH-activity curve, it is necessary to determine both the pH-Michaelis constant and pH-maximum velocity curves (see Dixon, 1953). In the final analysis it is also necessary to consider the effects of pH on the stability of the enzyme, the ionization of the substrate, and the state of aggregation of the enzyme molecules. It is hoped to investigate further some of these points at a later date. However, some points as far as the present results are concerned might be mentioned.

It is interesting that the buffers which depend on anions for their buffering capacity shift the pH optimum to the acid side of the pH optimum obtained in the absence of buffer. Cationic buffers, on the other hand, shift the pH optimum to the alkaline side. It is also noteworthy that the pH optimum in each buffer lies at the end of the alkaline range of the buffer, so that it was not possible to follow the alkaline branch of the pH-activity curve to an appreciable extent.

It is significant that the pH optimum of aconitase in catalyzing the three reactions *iso*citric acid→citric acid, *iso*citric acid→*cis*-aconitic acid and *cis*-aconitic acid→citric acid is the same. This would seem to indicate that not only was the same enzyme responsible for catalyzing these reactions, but also that the properties of the active centre(s) were similar. It might be supposed from these results that the same catalytic centre(s) was concerned in carrying out all the reactions associated with aconitase activity.

The inactivation of aconitase by buffers in the absence of substrate is likely to be a non-ionic reaction on account of the relatively slow rate of the inactivation. In the case of the inactivation by phosphate, it could be due to the interaction of the free $Fe^{2+}$ with the phosphate so as to upset the equilibrium between the free enzyme, free $Fe^{2+}$ and the $Fe^{2+}$-enzyme complex. The attainment of the new equilibrium would be a slow process. The ionic reaction between phosphate and the free $Fe^{2+}$ would conceivably be rapid, but the rate at which the $Fe^{2+}$ was removed from the enzyme would be slow. This reaction is the reverse of the activation process, which takes a measurable amount of time (Morrison, 1953). However, the above theory would not hold in the case of N-ethylmorpholine, as this buffer does not form co-ordination compounds with $Fe^{2+}$. As the rate of inactivation increases with a rise in the pH, it seems more likely that the inactivation of the enzyme is not concerned specifically with $Fe^{2+}$, but with a number of groups on the enzyme surface.

The ability of the substrates of the enzyme to prevent the inactivation which occurs in these buffers must be due to the fact that the enzyme has a greater affinity for the substrates than it has for the buffer ions. Nevertheless, according to the theory of enzyme action postulated by Michaelis and Menten (1913), there is always a small amount of enzyme which is not combined with substrate. Therefore, it is likely that the substrate protects the enzyme to a large extent, but not completely.

## SUMMARY.

It has been shown that the pH optimum of aconitase is influenced by the ions present in solution.

The pH optimum of aconitase in catalyzing the reactions *iso*citric acid→citric acid, *iso*citric acid→*cis*-aconitic acid and *cis*-aconitic acid→citric acid is the same

in the presence of a particular buffer and altered to the same extent in different buffers.

The inactivation of aconitase by buffers in the absence of substrate has been shown.

The significance of these findings has been discussed.

*Acknowledgments.* It is a pleasure to express my thanks to Sir Rudolph Peters, F.R.S., and to Dr. R. B. Fisher for their advice and interest in the work. Thanks are also due to Miss S. Read for skilled technical assistance.

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# A STUDY ON THE HAEMAGGLUTININ OF THE NAKAYAMA STRAIN OF JAPANESE B ENCEPHALITIS VIRUS

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Since Hirst in 1941 reported the agglutination of mammalian and avian red cells by influenza virus, an increasing number of viruses has been found to possess haemagglutinins for the erythrocytes of different animals (Rivers, 1952). Amongst the most recently discovered ones are those of St. Louis encephalitis (Chanock and Sabin, 1953), Murray Valley encephalitis (MacDonald, 1952), and Japanese B encephalitis. Sabin and Buescher (1950), who described the agglutination of one-day-old chick red cells by this latter virus, recommended its preparation by the extraction of infected mouse brain in unbuffered saline. A subsequent centrifugation at 13,000 r.p.m. for 1 hour was considered essential to remove a normal tissue inhibitor which adversely affected haemagglutinin storage. Later work by MacDonald (1952) on the allied virus of Murray Valley encephalitis demonstrated, however, that a considerable degree of haemagglutinin stability could be achieved in the supernate from one slow-speed centrifugation followed by storage at —20° C. The same author also reported that pigeon erythrocytes could be substituted for one-day-old chick cells in titrations with the above viruses.

With a view to using the Japanese B haemagglutination inhibition test as a diagnostic procedure, it seemed desirable to attempt to purify the haemagglutinin and to determine its relationship to the infectious particle and to the complement-fixing antigen.

## Materials and Methods.

*Virus.* The Nakayama strain of Japanese B encephalitis was obtained in 1949 from the Hooper Foundation for Medical Research, University of California. Virus from the 9th to the 11th mouse brain passages was used in the present work. As preliminary experiments confirmed the findings of Casals *et al.* (1951), that higher virus yields resulted from the use of mice younger than 3 weeks old, 8-day-old mice were routinely used for both haemagglutinin production and for infectivity titrations. Brains were removed from moribund mice and ground in unbuffered saline to a 20 p.c. suspension using sterile sand as an abrasive. The supernate resulting from centrifugation at 2,500 r.p.m. for 10 minutes represented the stock virus. When required, high speed centrifugation was carried out on a Spinco model L preparative ultracentrifuge, all runs being made at a temperature of 4° C.

*Antiserum.* Guinea-pig hyperimmune antiserum was prepared from an egg-adapted line of the Nakayama virus by intraperitoneal injection of 0·2 ml. of 20 p.c. infected chicken-embryo. This was followed on the 10th and 20th days by 0·15 ml. of the same antigen given by the intracerebral route. The animals were bled out on the 33rd day after commencement of immunization.

*Solutions.* Saline: (a) Unbuffered: 0·9 p.c., sodium chloride. (b) Buffered: 0·9 p.c. sodium chloride buffered to pH 6·7 with 0·1 M. phosphate buffer.

Buffers: Standard phosphate (pH 5·9 to 8·0) and veronal (pH 6·8 to 8·0) buffers at 0·1 molarity.

*Red Cells.* Pigeons were bled from a wing-vein into Alsever's solution. Experiments showed that the haemagglutinating ability of these cells was unchanged after storing in either Alsever's solution or in saline over a period of 10 days at 4° C. Fresh cells were therefore obtained at weekly intervals. In preparation for the test, cells were washed three times in buffered saline and finally suspended to a concentration of 0·5 p.c. in the same medium.

*Haemagglutination and haemagglutination-inhibition tests.* Doubling serial dilutions of virus were made in buffered saline in 0·25 ml. volumes. An equal volume of red-cell suspension was added to each tube and the test was read by the Salk (1944) pattern method after 1 hour at 4° C. The tube containing least virus and giving partial agglutination was regarded as containing 1 agglutinating dose (AD) and as the end-point of the titration.

Inhibitors, which were present in normal mouse brain, and antibodies, which were present in hyperimmune serum, were titrated by permitting serial 2-fold dilutions of the inhibitor or antibody (0·25 ml.) to react with an equal volume of virus diluted to contain 4 AD's. A further volume of 0·5 p.c. pigeon red cells was then immediately added to each tube and the series read after 1 hour at 4° C. The end-point was taken as the highest dilution of brain or serum (prior to the addition of virus and red cells) which showed complete inhibition of haemagglutination. The removal of certain non-specific serum inhibitors was effected by ether treatment (MacDonald, 1952).

*Infectivity titrations.* The virus was diluted in broth in 10-fold dilutions, using a separate pipette for each dilution, and 0·01 ml. inoculated intracerebrally into 8-day-old mice. As a routine procedure three mice were used for each dilution. Deaths were recorded over a period of 10 days and the $LD_{50}$ calculated according to the method of Reed and Muench (1938).

*Complement fixation test.* Serial 2-fold dilutions of each antigen (0·25 ml.) were mixed with an equal volume of a constant dilution of antiserum containing 4 units as measured by a box-titration. Two volumes of complement diluted to contain 2 units were added. After shaking, the tubes were held at 4° C. overnight and subsequently for 30 minutes in a 37° C. water-bath. Sensitized sheep red cells (2 volumes) were added, the racks shaken, and the tests read after a further 30 minutes at 37° C. The end-point was taken as the serum dilution giving partial (approximately 50 p.c.) haemolysis.

## EXPERIMENTAL.

### *Performance of the haemagglutination test.*

MacDonald (1952) recommends refrigeration of reagents and tubes before diluting the virus and adding the red cells; however, preliminary tests showed that with Japanese B virus and using pigeon red cells this was unnecessary.

TABLE 1.

*Influence of temperature on titration of Japanese B haemagglutinin.*

| Reagents and tubes | Dilutions prepared at | Titration conducted at 4° C. | Titration conducted at 19° C. |
|---|---|---|---|
| Chilled prior to dilution | 4° C. | 9,600 | 3,200 |
| | 19° C. | 12,800 | 3,200 |
| *Not* chilled prior to dilution | 4° C. | 12,800 | 1,600 |
| | 19° C. | 19,200 | 800 |

These titrations were carried out employing a 13,000 r.p.m. supernate similar to that used by Sabin and Buescher (1950), where, as shown in Table 1, it was obvious that although the end-points were higher when the tests were held at refrigeration temperature, both the prechilling of reagents and the use of ice-water to surround dilution tubes were unnecessary refinements.

The titration procedure finally adopted was to make all virus dilutions and red cell additions at room-temperature, followed by refrigeration for 1 hour before reading.

The specificity of viral haemagglutination was shown by its inhibition by Japanese B virus hyperimmune serum, but not by Western Equine antiserum. Under haemagglutination test conditions, a normal mouse brain suspension failed to agglutinate pigeon red cells.

*Stability of haemagglutinin.*

There was considerable variation in stability of the haemagglutinin when prepared according to the method of Sabin and Buescher (1950) and stored at 4° C. Despite apparently identical conditions of preparation and storage, 17 samples, prepared over a period of 3 months, fell into two groups:

(a) Those in which the titre decreased fairly rapidly and resulted in little or no haemagglutinin being detectable after 2 months.

(b) Those in which the titre remained at about the original level for 4 months or longer.

In no case was there any marked enhancement of the titre after storage at 4° C. for 1 week, as reported by Sabin (1951), and by Sabin and Buescher (1950).

The pH of the suspending medium has a definite effect on the stability of the haemagglutinin when stored at 4° C. When buffered below pH 6·7 the haemagglutination titre dropped from 1:12,800 to 1:400 within 1 hour, while in preparations buffered at pH 6·7 the drop was only 2 tubes in 1 hour (1:12,800 to 1:3,200). In the latter instance deterioration continued until after 24 hours the titre was 1:800. Stored at pH 7·0 to 7·6, the drop in titre was only 1 or 2 tubes even after 16 days.

Heating at 54° C. for 5 minutes was sufficient to destroy all the haemagglutinin.

To determine possible methods whereby a stable haemagglutinin may be prepared, infected mouse brains were extracted by five different techniques and stored in rubber-stoppered tubes at 4° C. and —20° C. At intervals over a period of 2 months they were titrated against pigeon cells.

*Preparation of antigens.*

(1) Infected mouse brains were extracted to form a 20 p.c. suspension in unbuffered saline grinding with sterile sand and centrifuging at 2,500 r.p.m. for 10 minutes (MacDonald, 1952).

(2) The supernate resulting from centrifuging preparation (1) at 13,000 r.p.m. for 60 minutes at 4° C. (Sabin and Buescher, 1950).

(3) A mixture of equal parts of preparation (2) and buffered saline pH 6·8 was adsorbed with 10 p.c. by volume of calcium phosphate gel for 1 hour at 4° C. The supernate was discarded and the gel resuspended in a fresh virus preparation and adsorption repeated. After washing the calcium phosphate twice in chilled saline, it was mixed with 10 volumes of buffered saline pH 7·7 and the virus permitted to elute at room temperature for 2 hours.

(4) Equal parts of preparation (2) (chilled) and chilled 20 p.c. methanol were mixed slowly in a flask in an ice-bath. The mixture was held at 4° C. for 3 hours, after which it was centrifuged at 3,500 r.p.m. for 30 minutes at 4° C. The supernate was replaced with a volume of buffered saline pH 7·7 equal to that of the original virus suspension, and solution effected by mixing and holding at room temperature for 30 minutes. The supernate, after centrifuging at 2,000 r.p.m. for 15 minutes, represented the final virus preparation.

(5) Infected mouse brains were extracted at room temperature with acetone and ether (Casals and Brown, 1953) and the dried residues leached with unbuffered saline at 4° C. to give a 20 p.c. suspension of the original brain weight.

The original titres and changes over a storage period of 2 months are given in Table 2.

TABLE 2.

*Stability of Japanese B haemagglutinin preparations.*

| Antigen preparation | 1 | | 2 | | 3 | | 4 | | 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Storage temperature ° C. | 4 | −20 | 4 | −20 | 4 | −20 | 4 | −20 | 4 | −20 |
| Original titre | 2,560 | | 2,560 | | 320 | | 80 | | 40 | |
| After 1 month | 5,120 | 2,560 | 5,120 | 2,560 | 320 | 80 | 80 | <10 | <10 | 10 |
| After 2 months | 2,560 | 2,560 | 2,560 | 2,560 | 160 | <10 | 160 | <10 | <10 | 40 |

As shown in Table 2, the initial haemagglutinin titres of antigens (3), (4) and (5) were considerably reduced when compared with those prepared by simple saline extraction. The latter antigens (1) and (2) were also more stable at both 4° C. and —20° C. over an examination period of 2 months. On the whole, refrigeration temperature (4° C.) favoured the haemagglutinin stability of antigens partially purified by chemical methods. However, even under the most favourable conditions, the working titres of these latter preparations were considered inadequate for routine use.

*Ultracentrifugation.*

Ultracentrifugation at 30,000 r.p.m. (90,000×*g.*) was tried as a possible means of distinguishing between the haemagglutinin, the complement-fixing antigen, and the infectious unit. Analysis of the deposit resulting from 1 hour at this speed showed the latter two components to be almost quantitatively deposited. The haemagglutinin content was, however, disproportionate to that contained in the original suspension and its reduced titre could only be accounted for by inactivation during the process of centrifugation. A limited number of separation attempts employing a sucrose gradient gave equivocal results, and the method therefore was not persisted with.

*Factors influencing haemagglutination.*

(a) *Concentration of red cells.* Varying the concentration of pigeon red cells between 0·25 p.c. and 2 p.c. produced a proportionate decrease in the haemagglutination titre comparable with that found by Sabin and Buescher (1950) using 1-day-old-chick cells.

(b) *pH and molarity.* By titrating the viral preparation in saline buffered at different pH levels and plotting the observed haemagglutination titre against pH, a curve was obtained almost identical with that of Sabin and Buescher (1950). Maximal titres occurred between pH 6·4 and pH 6·7. Molarity of the buffer used in the test made little difference to the end-point over the range 0·1 M to 0·0005 M. The actual buffering agent also seemed of minor importance since veronal and phosphate could be interchanged with no appreciable variation in titre.

*Adsorption and elution of haemagglutinin.*

The relative ease with which Japanese B virus haemagglutinated pigeon red cells at refrigeration temperature indicated that this technique might well prove satisfactory for virus concentration. However, a preliminary study of adsorption rates at 4° C. indicated that despite buffering over the narrow pH spectrum necessary for optimal adsorption (pH 6·7 to 6·9), the amount of haemagglutinin removed from a standard virus suspension varied considerably. In cases where no residual haemagglutinin could be detected in a single virus suspension following repeated adsorption with fresh pigeon red cells, both the complement-

fixing antigen and infectious particles were present, but in reduced concentration (Table 3). Pigeon red cells, saturated with haemagglutinin by several exposures to fresh virus in the cold, commenced elution of this component after remaining at room temperature (19° C.) for 1 hour. The suspending fluid consisted of saline buffered over a pH range of 6·8 to 8·0 (optimal 7·7) at 0·01 M. Elution proceeded for at least 20 hours under the above conditions, the total recovery of haemagglutinin representing 25 p.c. of that in the original suspension. Similarly buffered controls of the latter preparation showed the pH employed to be optimal for haemagglutinin preservation at room temperature.

TABLE 3.

*Adsorption and elution of Japanese B virus.*

| Adsorbant | Component | Original material | Supernate after adsorption | Eluate (2 hours) | Eluate (20 hours) |
|---|---|---|---|---|---|
| Pigeon cells | HA | 2,560 | 20 | 320 | 320 |
| | CF | 240 | 160 | N.D. | N.D. |
| | $\frac{1}{\log}$ $LD_{50}$ | 8·0 | 6·5 | 5·5 | 4·5 |
| Calcium phosphate | HA | 2,560 | <20 | 1,280 | 640 |
| | CF | 240 | <20 | <20 | <20 |
| | $\frac{1}{\log}$ $LD_{50}$ | 8·0 | 6·2 | 5·5 | < 3·0 |

HA=haemagglutinin. CF=complement-fixing antigen. N.D.=not done.

As the interaction of haemagglutinin with pigeon cells had every indication of being non-specific in character, several inorganic compounds (calcium phosphate, celite, Fuller's earth and calcium carbonate) were examined for their adsorptive properties. Of these, calcium phosphate seemed the most promising and was found capable of completely adsorbing the haemagglutinin and complement-fixing antigen from a chilled suspension buffered over a pH range of 6·9 to 8·0. Virus subjected to identical conditions of temperature and pH, but containing no adsorbant, gave essentially the same haemagglutinin, complement-fixing antigen and infectivity titres as those of the original material. As with virus-adsorbed pigeon red cells, elution commenced promptly at room temperature into saline buffered at the optimal pH of 7·7 (0·01 M.). This was rapid, and by 2 hours maximum yields of haemagglutinin and infectious particles were present (Table 3). Complement-fixing antigen, which had been initially completely adsorbed onto the carrier particles, failed to elute even after prolonged incubation.

*Mouse brain haemagglutination inhibitor.*

Infected mouse brain preparations have been found to contain a component capable of inhibiting Japanese B virus haemagglutination (Sabin and Buescher, 1950). In the preesnt work such activity was often manifest by abnormally granular red cell sedimentation patterns in titration tubes containing high concentrations of infected mouse brain. However, the small amount of inhibitor demonstrable under such conditions was rapidly diluted out and in no way affected the final virus haemagglutination titre. Employing a standard haemagglutination-inhibition test against 4 AD's of Japanese B virus, normal mouse brain inhibitor titres of 1:80 to 1:160 were regularly obtained. These figures were unchanged by either the animal age (range tested 9 to 86 days) or by an active infection with the homologous virus. Similar results were obtained following incubation *in vitro* of virus and normal mouse brain suspension at 37° C. for 4 hours or longer. The inhibitor withstood a temperature of 54° C. for 5 minutes, but suffered a 2-fold titre reduction after 30 minutes. Thus the masking effect of viral haemagglutination upon low concentrations of mouse-brain inhibitor could be successfully removed by inactivation of the former component by brief exposure at this temperature. Normal mouse-brain inhibitor could be satisfactorily extracted with either distilled water (pH approximately 6·8 to 7·0) or unbuffered saline, and over a pH range of 6·8 to 8·0 remained fully active at 4° C. for at least a week. Intracerebral neutralization tests carried out in mice with mixtures of normal mouse-brain extracts and Japanese B virus (1 hour at 4° C. before injection) showed the latter to possess no sparing effect.

## DISCUSSION.

The haemagglutinin in mouse brains infected with the Nakayama strain of Japanese B encephalitis and detectable with 1-day-old-chick cells (Sabin and Buescher, 1950) can also be demonstrated with adult pigeon cells. It may be extracted by unbuffered saline or by distilled water or by the acetone-ether method of Casals and Brown (1953). Simple saline extraction, however, results in a product possessing a higher titre and greater stability than the other two methods. Although haemagglutination occurs at room temperature, the end-point is significantly higher and more clear-cut when titrations are conducted at 4° C. However, it is unnecessary to pre-chill the reaction tubes and reagents or to carry out the virus dilutions in an ice-bath as has been reported previously (MacDonald, 1952). Under such conditions reproducible haemagglutination titres could be consistently obtained. No difficulty was experienced in reading end-points, since the agglutinated shield of red cells remained stable for many hours. Even thorough shaking of the test racks, followed by sedimentation at room temperature, failed to change the original titre. Thus elution of haemagglutinin adsorbed upon pigeon cells is apparently not active at temperatures between 4° C. and 19° C.

The development of stable haemagglutinin suspensions has not been altogether satisfactory. Preliminary experiments, extending over 2 months and using crude infected mouse-brain suspensions, suggested that workable titres could be expected for only 7 days after preparation and storage at either 4° C. or —20° C. However, with no obvious change in technique, perfectly stable antigens were realised and continued to be regularly produced throughout the latter 6 months of this study. Attempts to obtain purified suspensions of haemagglutinin indicated that while these might be obtained either by adsorption onto and elution from an inert carrier particle or by methanol precipitation, their reduced titres and rapid deterioration upon storage made them inferior to brain extracts clarified by simple ultracentrifugation. Results obtained during this study have suggested that the complement-fixing antigen may be separated from the haemagglutinin and infectious property by the former's inability to elute from a calcium phosphate-virus complex. While it is admitted that considerable amounts of complement-fixing antigen may have remained undetected due to the lack of sensitivity inherent in this test, it would seem that the presence in measurable quantities of this component is unnecessary for either the processes of infection or of haemagglutination. On the other hand, attempts satisfactorily to separate the two latter fractions have failed, an observation which substantiates the suggestion of Sabin and Buescher (1950) that the haemagglutinin is in reality a polymerized form of the infectious particle.

## SUMMARY.

A study has been made of the physical conditions necessary for the agglutination of pigeon red cells by the Nakayama strain of Japanese B encephalitis virus. Satisfactory antigens capable of prolonged storage at either refrigeration or sub-freezing temperatures may readily be produced from the infected brains of 9-day-old mice. Attempts to purify the viral haemagglutinin by adsorption and elution with calcium phosphate have suggested that the complement-fixing antigen is distinct from both the haemagglutinin and the infectious particle.

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# "FREEZE-DRYING" WITH CALCIUM CARBIDE

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Where solid carbon dioxide ("dry ice") is readily available there can be but little doubt that it is the most convenient means of trapping the water evaporated from biological material that is being dried from the frozen state *in vacuo* down to the lowest pressure (a few microns) so obtainable with the use of the two-stage rotary pump. Many workers are, however, engaged in biological studies in laboratories situated inconveniently far from the nearest supply of "dry ice." These workers, it is hoped, may find the apparatus and procedure described below useful for the "freeze-drying" of small specimens of anti-sera and of living micro-organisms. In this Institute the method has resulted in the same final pressure, 5 microns, which is obtained with "dry ice" and appears to be limited by the materials of which the apparatus is constructed and by the pump employed. The method conforms to the following very desirable conditions (c.f. Thorne, 1951).

When living organisms are dried, the cotton-wool plugs remain in the ampoules throughout the process.

The apparatus is not evacuated until after the material is frozen.

Air is not admitted to the ampoules at any stage.

The reagent used for absorption of the water is calcium carbide. This substance, carefully screened and substantially free from dust, is sold in small air-tight tins for the use of anglers and campers and requires no further breaking or other treatment to fit it for use. Its condition is easily ascertained by a shake of the tin when a clear, unmuffled sound shows that the carbide is clean and free from powdered calcium hydroxide.

The apparatus comprises the following portions: vacuum pump, pirani gauge, absorption tube, manifold, ampoules and a 1-gallon vacuum jar. The pump, gauge, ampoules and vacuum jar have been previously described (Holden and Scott, 1951). For especially delicate organisms a pump with a higher speed and, perhaps, a rather lower ultimate pressure might be an advantage. The absorption tube is of "Pyrex" glass 65 cm. long, 2·5 cm. bore, fitted at the end nearest the pump with a short piece of glass tube 7–8 mm. outside diameter and, at the other, joined to the manifold with a B29 ground joint. On account of its vibration, the pump is joined to the absorption tube with a piece of red rubber

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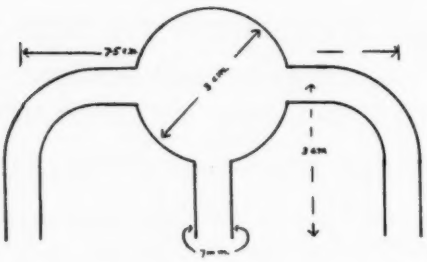

**Fig. 1.** Cross-section of manifold to show the disposition of the side-tubes. The four groups of side-tubes are 30 mm. apart, measured along the manifold.

"pressure" tube 6 mm. bore and about 15 cm. long. The manifold (Fig. 1) is also made of "Pyrex" glass tube 30 cm. long and about 3 cm. outside diameter. One end carries the B29 joint to unite it to the absorption tube; at the other is a short length of tube for the rubber connection to the gauge and a capillary tap for the slow admission of air to the system after use. The manifold has 12 side-tubes for the connection of ampoules.

*Procedure.* In the end of the absorption tube nearest the pump is placed a pad of loosely-packed cotton-wool 5 cm. thick to protect the pump from damage by abrasive dust. The next 50 cm. of the tube is filled with calcium carbide poured from the tin, no attempt being made to pack it tightly with a rammer. Another pad of cotton-wool is put in after the carbide and the ground joint carefully wiped free from dust. The joint is greased with a good white petroleum jelly, the manifold connected and the rubber tube to the pump slipped on. The tap is closed, the gauge connected to the manifold and the ampoules to the rubber tubes on its side-tubes. In the vacuum jar is placed a mixture of crushed ice and salt to within 5 cm. of the top and the interstices are filled with ice-cold brine. The absorption tube and manifold are supported on a stand at such a height above the bench that the bottoms of the ampoules are above the level of the rim of the vacuum jar. The jar is raised until the ampoules are immersed in the freezing mixture to within about 2 cm. of the bottoms of their rubber tubes and is so supported on blocks.

After 30 minutes, to allow for freezing, a clip is placed on the rubber tube between the pump and the absorption tube. The clip is closed, the pump started and the clip opened slowly to avoid undue agitation of any dust in the apparatus. Within 3–4 minutes the clip should be fully opened and the gauge is then switched on. The temperature of the ice-salt bath should be below —10° C., preferably —12° to —13° C. After 24 hours, when drying is nearly complete, the vacuum jar is removed and the ampoules rinsed with water and gently wiped without disconnecting them. For a further 24 hours drying is allowed to

proceed with the ampoules at the temperature of the laboratory. The manifold is then rotated about the ground joint till the ampoules are horizontal; they are then sealed with a small sealing burner with two impinging jets made of "Pyrex" glass. The cotton-wool plugs in the ampoules containing living organism must *not* be removed. The drying proceeds through the plugs and the risk of contamination is very much less. Should more than one type of organism or serum be dried at one time, the ampoules must be marked with a diamond pencil below the constriction. The inside of all the rubber tube used must be carefuly brushed with hot soap and water and well rinsed before attaching it to the apparatus. Glass-rubber joints which are not to be dismantled may be painted outside with a little shellac in alcohol.

The freeze-drying should be performed in a fume-chamber or a well ventilated room. Perhaps it may be added that, like anything that can react with water-vapour at a low temperature, calcium carbide is a dangerous substance. It must not be left in the closed absorption tube. An attractive feature of this process is that nearly all the reaction occurs right at the "manifold" end of the absorption tube and the rest of it serves to trap the last traces of water. The spent powder is easily discarded and the remaining carbide can be returned to a storage tin. Large tins of carbide should never be used. If carbide is purchased in drums it should be carefully transferred to an appropriate number of 1 lb. tins and the latter carefully closed and sealed.

The vacuum jar is essential; attempts to substitute an insulated vessel of a less costly and fragile type were unsuccessful. A duplicate jar, manifold and absorption tube are advisable. The pump motor should be chosen for its ability to endure long periods of hard work at the highest temperature met with in the laboratory concerned.

## SUMMARY.

The use is described of calcium carbide to absorb water-vapour in the "freeze-drying" of small amounts of biological material.

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